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Adenosine A_{2A} Receptors in the Amygdala Control Synaptic Plasticity and Contextual Fear Memory

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The consumption of caffeine modulates working and reference memory through the antagonism of adenosine A_{2A} receptors (A_{2A}Rs) controlling synaptic plasticity processes in hippocampal excitatory synapses. Fear memory essentially involves plastic changes in amygdala circuits. However, it is unknown if A_{2A}Rs in the amygdala regulate synaptic plasticity and fear memory. We report that A_{2A}Rs in the amygdala are enriched in synapses and located to glutamatergic synapses, where they selectively control synaptic plasticity rather than synaptic transmission at a major afferent pathway to the amygdala. Notably, the downregulation of A_{2A}Rs selectively in the basolateral complex of the amygdala, using a lentivirus with a silencing shRNA (small hairpin RNA targeting A_{2A}R (shA_{2A}R)), impaired fear acquisition as well as Pavlovian fear retrieval. This is probably associated with the upregulation and gain of function of A_{2A}Rs in the amygdala after fear acquisition. The importance of A_{2A}Rs to control fear memory was further confirmed by the ability of SCH58261 (0.1 mg/kg; A_{2A}R antagonist), caffeine (5 mg/kg), but not DPCPX (0.5 mg/kg; A_1R antagonist), treatment for 7 days before fear conditioning onwards, to attenuate the retrieval of context fear after 24–48 h and after 7–8 days. These results demonstrate that amygdala A_{2A}Rs control fear memory and the underlying process of synaptic plasticity in this brain region. This provides a neurophysiological basis for the association between A_{2A}R polymorphisms and phobia or panic attacks in humans and prompts a therapeutic interest in A_{2A}Rs to manage fear-related pathologies.

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INTRODUCTION

The encoding of fear-related memory is well established to involve abnormal plastic changes of information processing in amygdala circuits (Johansen *et al*, 2011; Mahan and Ressler, 2012). In other brain regions, synaptic plasticity is controlled by the adenosine neuromodulation system (Fredholm *et al*, 2005), which involves a coordinated action of inhibitory A_1 receptors (A_1Rs) and facilitatory A_{2A} receptors (A_{2A}Rs) to fine tune brain neurotransmission (Cunha, 2008). In hippocampal circuits, A_{2A}Rs are found in synapses (Rebola *et al*, 2005a), namely in glutamatergic synapses (Rebola *et al*, 2005b), and are selectively engaged to control synaptic plasticity (Rebola *et al*, 2008; Costenla *et al*, 2011). The importance of this modulation system is best heralded by the observation that the overactivation of hippocampal A_{2A}Rs is necessary and sufficient to trigger spatial memory dysfunction (Li *et al*, 2015a; Pagnussat *et al*, 2015). Furthermore, conditions associated with memory deterioration trigger an upregulation of A_{2A}Rs in the hippocampus leading to abnormal synaptic plasticity (Costenla *et al*, 2011; Kaster *et al*, 2015), and A_{2A}R blockade prevent memory impairment in conditions such as stress, aging, or Alzheimer's disease (eg Batalha *et al*, 2013; Laurent *et al*, 2016; Oor *et al*, 2015; Prediger *et al*, 2005), an effect mimicked by caffeine (a nonselective adenosine receptor antagonist) both in animal models and in humans (reviewed in Cunha and Agostinho, 2010; Chen, 2014).

Interestingly, the acute administration of caffeine disrupts fear memory (Corodimas *et al*, 2000) and A_{2A}R polymorphisms are associated with panic disorders (Deckert *et al*, 1998; Hamilton *et al*, 2004), but it is unknown whether A_{2A}Rs control fear memory and synaptic plasticity in amygdala circuits. Thus, we now explored the involvement of A_{2A}Rs in the control of synaptic plasticity in the amygdala and their possible role in the control of fear memory.

MATERIALS AND METHODS

For detailed Materials and Methods, see 'Supplementary Methods'.

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Mice and Drug Treatments

All experiments were approved by the Ethical Committee of the Center for Neuroscience and Cell Biology (Orbea 78-2013). Male C57Bl/6 mice (2–3 months) were daily intraperitoneally injected either with caffeine (5 mg/kg; Sigma, Sintra, Portugal; a dose preventing memory deficits without altering locomotion; Prediger *et al*, 2005) or with supramaximal but selective doses of the A_1R antagonist DPCPX (1,3-dipropyl-8-cyclopenthylxanthine, 0.5 mg/kg; Tocris, Bristol, UK), or the $A_{2A}R$ antagonist SCH58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo-[1,5-*c*]pyrimidine; 0.1 mg/kg; Tocris), which are devoid of locomotor or nociceptive effects (Bastia *et al*, 2002), but effectively control neuronal dysfunction (Nakamura *et al*, 2002; Kaster *et al*, 2015). Drug treatments started 10 days before behavioral testing until the mice were killed.

Density and Localization of Adenosine Receptors

Western blot analysis with goat or mouse anti-$A_{2A}R$ antibodies (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA or Millipore, Madrid, Spain, respectively), which selectivity was confirmed by the lack of signal in $A_{2A}R$-knockout (KO) mice (Rebola *et al*, 2005a), or receptor binding analysis with 3 nM of ^{3}H-SCH58261 (specific activity of 77 Ci/mmol; prepared by GE Healthcare and offered by Dr E Ongini, Schering-Plough, Italy) or 6 nM of ^{3}H-DPCPX (specific activity of 109.0 Ci/mmol; DuPont NEN, Boston, MA, USA) was carried out in total membranes and membranes from synaptosomes (Costenla *et al*, 2011; Kaster *et al*, 2015), whereas the immunocytochemical detection of $A_{2A}Rs$ in glutamatergic nerve terminals was carried out as described previously (Costenla *et al*, 2011; Rebola *et al*, 2005b), using goat anti-$A_{2A}R$ (1 : 200; Santa Cruz Biotechnology) and guinea-pig anti-vesicular glutamate transporter type 1 (vGluT1; 1 : 1000, Chemicon, Temecula, CA, USA) antibodies.

Electrophysiological Recordings in Corticoamygdala Synapses

Electrophysiological recordings in brain slices were carried out as described previously (Costenla *et al*, 2011) by extracellularly recording population spikes in the lateral nuclei of the amygdala upon stimulation of the external capsule (EC). Long-term potentiation (LTP) was induced with three pulses of 100 Hz delivered with an interval of 5 s.

Generation and Administration of Lentiviral Vectors

An $shA_{2A}R$ (nts 419–437; see Figure 3) was inserted into a lentivector together with an enhanced green fluorescent protein (EGFP) reporter gene, as described previously (Alves *et al*, 2008). A hairpin designed to target the coding region of red fluorescent protein (nts 22–41) was used as an internal control (sh-control). These lentivectors (1 μl at 750 000 ng of p24 antigen per ml) were stereotaxically delivered at an infusion rate of 0.1 μl/min in the following coordinates: anteroposterior: −1.1 mm; lateral: ±2.8 mm; ventral: −4.6 mm, and the injection site was confirmed on killing of the mice. $A_{2A}R$ downregulation was probed after 3 weeks by qPCR.

Auditory Fear Conditioning

Fear conditioning was performed as described previously (Goosens *et al*, 2000) in context A with three presentations of an auditory conditioned stimulus (CS; 80 dB for 30 s at 4 kHz) paired with a footshock unconditioned stimulus (US; 0.3 mA for 2 s, delivered 28 s after the beginning of CS) with a 60 s intertrial interval. At days 2 and 8, mice were returned to context A to test their freezing behavior for 8 min. At days 3 and 9, mice were placed in a different chamber (context B), the CS was presented after 3 min, and the freezing behavior was measured for 8 min.

Other Behavioral Analyses

The spontaneous locomotion of mice was measured 1 day after the fear conditioning protocols, in an open field test as described previously (Wei *et al*, 2014; Kaster *et al*, 2015). Nociceptive responses were evaluated 1 day after the open field test by using the hot-plate test (Le Bars *et al*, 2001).

Statistical Analysis

Results are presented as mean ± SEM. Behavioral data were analyzed with a one-way ANOVA followed by a Tukey's multiple comparison *post hoc* test or with a two-way ANOVA followed by Bonferroni *post hoc* tests, when more than one variable and condition (eg, genotype and time) were analyzed. Binding, western blot, and electrophysiological data were analyzed with unpaired Student's *t* tests. The significance level was 95%.

RESULTS

$A_{2A}Rs$ are Localized to Glutamatergic Terminals in the Amygdala

We first probed whether $A_{2A}Rs$ were located in glutamatergic synapses in the amygdala as occurs in the hippocampus (Rebola *et al*, 2005b). As shown in Figure 1a, the binding density of ^{3}H-SCH58261 was larger ($n=6$, $p<0.05$) in synaptosomal membranes (30.90 ± 3.47 fmol/mg protein) than in total membranes from the amygdala (20.75 ± 2.13 fmol/mg protein). Furthermore, the density of $A_{2A}Rs$ as evaluated by western blot was also 18.6 ± 5.2% larger ($n=6$, $p<0.05$) in synaptosomal compared with that in total membranes from the amygdala (Figure 1b), showing that $A_{2A}Rs$ are indeed enriched in amygdala synapses. A double immunocytochemical labeling of $A_{2A}Rs$ and of a glutamatergic marker ($vGluT_1$) in amygdala nerve terminals (Figure 1c) revealed that 40.4 ± 3.5% ($n=5$) of the $vGluT_1$-positive terminals were endowed with $A_{2A}Rs$ (arrows indicate regions of overlap). Overall, these findings show that $A_{2A}Rs$ are present in the amygdala, and found in glutamatergic synapses.

$A_{2A}Rs$ Control Synaptic Plasticity in the Amygdala

Changes in synaptic transmission in the amygdala are thought to underlie the acquisition and expression of long-term fear memories (Blair *et al*, 2001; Goosens and Maren, 2001; Johansen *et al*, 2011). Thus, we next tested the ability of $A_{2A}Rs$ to control synaptic plasticity in excitatory synapses in

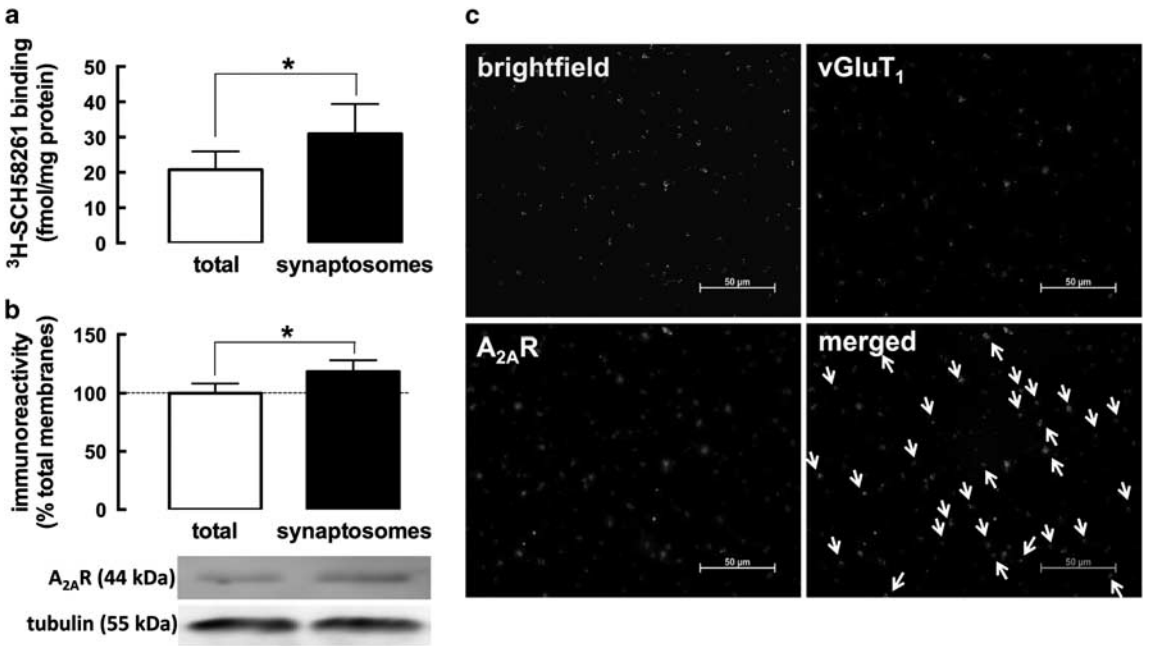


Figure 1 Adenosine A_{2A} receptors (A_{2A}Rs) are enriched in synapses and located in glutamatergic synapses in the amygdala. The comparison of the binding of a supramaximal concentration of a selective A_{2A}R antagonist, ^{3}H-SCH58261 (^{3}H-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo-[1,5-c]pyrimidine, 3 nM) (a) or of the immunoreactivity of A_{2A}Rs (b) was larger in membranes from synaptosomes (purified synapses) than in total membranes of the amygdala. Data are mean ± SEM of six mice; *$p<0.05$, unpaired Student's *t*-test. Representative photographs of an immunocytochemical analysis of purified nerve terminals from the amygdala (c), which revealed a colocalization (yellow) of A_{2A}R immunoreactivity (green) in nerve terminals immunopositive for vGluT1 (vesicular glutamate transporters type 1, a marker of glutamatergic nerve terminals; red), as indicated by the arrows (scale bar: 50 µm). This experiment is representative of five experiments with similar results. The selectivity of the A_{2A}R antibody was confirmed by the lack of western blot and immunocytochemical signal in synaptosomes from A_{2A}R knockout mice. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

the lateral amygdala, one of the primary sites of CS–US (conditional-unconditional stimuli) convergence during fear conditioning, upon stimulation of afferents in the EC in slices. The bath superfusion with SCH58261 (50 nM) failed to modify both basal neurotransmission (Figure 2a) and short-term plasticity (ie, paired pulse stimulation; Figure 2b). Instead, SCH58261 (50 nM) selectively impaired synaptic plasticity, shown by the reduction of LTP amplitude (128.8 ± 6.8% potentiation over baseline in the presence of SCH58261 compared with 171.0 ± 13.3% in its absence, $n=6$, $p<0.05$) (Figure 2c). Another selective A_{2A}R antagonist, ZM241385 (50 nM), also reduced LTP amplitude (119.3 ± 12.9%) compared with control (160.2 ± 12.1%, $n=5$, $p<0.05$) (Figure 2e).

Downregulation of A_{2A}Rs in the Basolateral Amygdala Impairs Long-Term Fear Memory

As the basolateral complex of the amygdala is a key circuit for the expression of fear memories through alterations of synaptic plasticity (Blair *et al*, 2001; Goosens and Maren, 2001; Johansen *et al*, 2011), we tested if the selective elimination of A_{2A}Rs in the amygdala was sufficient to affect conditioned fear. We developed lentivectors encoding shRNAs to selectively neutralize A_{2A}Rs (shA_{2A}R) while simultaneously expressing EGFP (Figure 3a). These lentivectors have neuronal tropism and limited spread in the brain parenchyma (Lundberg *et al*, 2008). The lentivirus infected only neurons (colocalization with NeuN and no colocalization with GFAP, data not shown, see Viana da Silva *et al*, 2016), and covered the majority (71.0 ± 8.8%, $n=4$) of the basal nucleus and spread only to the lateral nucleus of the amygdala (7.56 ± 1.03%, $n=4$), as judged by the superimposable staining of EGFP (Figure 3b) and of acetylcholinesterase (Figure 3c), which is abundantly expressed in these nuclei (Berdel *et al*, 1996). The dissection of the amygdala 4 weeks after transfection revealed a near 70% decrease of A_{2A}R mRNA levels compared with amygdala tissue collected from sh-control mice (Figure 3d). An immunohistochemical confirmation of A_{2A}R protein levels could not be performed because A_{2A}R density in the amygdala is below the threshold of detection, although the striatal injection of the lentivector downregulated A_{2A}R protein by 55.4 ± 4.9% upon transfection of 27.2 ± 2.7% striatal neurons ($n=4$); instead, as previously shown in the hippocampus (Viana da Silva *et al*, 2016), we functionally confirmed the efficiency of shA_{2A}R lentivectors to downregulate amygdala A_{2A}Rs by showing that shA_{2A}R treatment abrogated A_{2A}R modulation of amygdala LTP: as shown in Figure 3e, SCH58261 was devoid of effects on LTP amplitude in slices from shA_{2A}R mice, but decreased LTP in slices from sh-control mice (Figure 3f); this enables using shA_{2A}R lentivectors to probe the involvement of amygdala A_{2A}Rs in fear memory.

Mice injected in the amygdala with shA_{2A}R had a similar spontaneous locomotion in the open field (Figure 3g) but displayed significantly less freezing during fear conditioning compared with sh-control mice (Figure 3h). ANOVA analysis of the time of freezing confirmed an effect of trials ($F_{2,16}=21.22$, $p<0.0001$), of the administration of shA_{2A}R

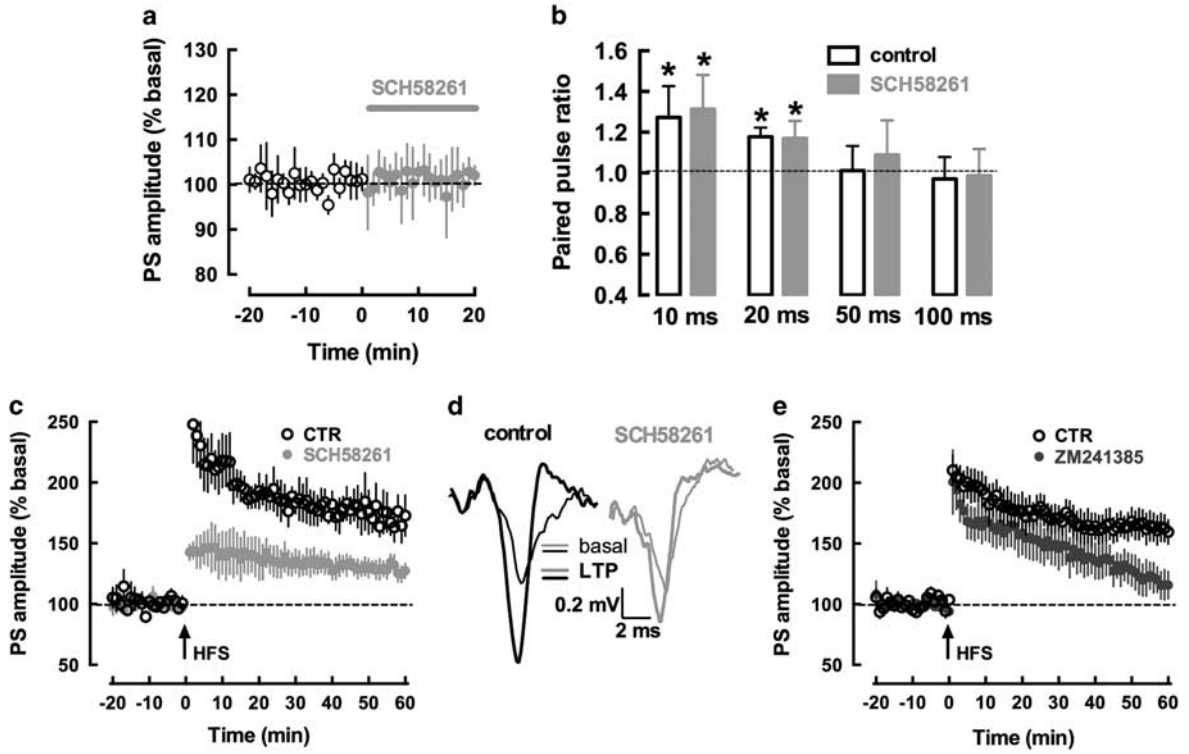


Figure 2 Adenosine A_{2A} receptors (A_{2A}Rs) selectively control long-term synaptic plasticity in the amygdala. Upon extracellular recording of population spike responses in the lateral amygdala triggered by stimulation of the external capsula, the selective A_{2A}R antagonist SCH58261 (amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo-[1,5-*c*]pyrimidine, 50 nM, in pink) did not change either basal synaptic transmission (a) or short-term plasticity evaluated as the paired pulse ratio with different interpulse intervals from 10 to 100 ms (b); in contrast, SCH58261 decreased the long-term potentiation (LTP) of the population spike responses triggered by a high-frequency stimulation (HFS) train (three pulses of 100 Hz delivered with an interval of 5 s), as shown in the time course (c) or in the pair of superimposed population spike responses before the train (baseline) and 60 min after HFS (d). Similarly, another chemically distinct but equally selective A_{2A}R antagonist ZM241385 (4-(2-[7-amino-2-(2-furyl{1,2,4}-triazolo{2,3-a{1,3,5}triazin-5-yl-aminoethyl}])phenol, 50 nM, in purple) also decreased LTP amplitude compared with its absence (control, black symbols) (e). Data are mean ± SEM of six to nine mice per group when testing SCH58261 and $n = 5$–6 when testing ZM241385. *$p < 0.05$ compared with the respective control (ie, lack of drugs, open symbols), unpaired Student's *t*-test. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

($F_{1,8} = 6.61$, $p < 0.05$), and a trial × shA_{2A}R interaction ($F_{2,16} = 7.45$, $p < 0.05$). During context re-exposure (Figure 3i), shA_{2A}R-treated mice froze less than control mice either the next day ($F_{1,4} = 19.87$, $p < 0.05$) or 7 days after fear conditioning ($F_{1,4} = 5.95$, $p < 0.05$). When tested for tone-induced fear (Figure 3j), shA_{2A}R-treated mice displayed a lower response at day 3 ($F_{1,4} = 6.51$, $p < 0.05$), and similar responses at day 9 ($F_{1,4} = 0.99$, $p = 0.39$). Notably, shA_{2A}R-treated animals performed similarly to control animals in two tests probing spatial reference memory, namely the two-trial Y-maze and the object displacement test (data not shown).

Adaptive Changes of the Adenosine Neuromodulation System After Fear Conditioning

We next determined whether alterations of amygdala A_{2A}R density and function accompany the plastic changes that occur in amygdala circuits during the implementation of fear memories. The density of A_{2A}Rs in amygdala membranes was higher ($p < 0.0001$, $n = 9$) in fear conditioned mice (33.0 ± 4.9 fmol/mg protein) compared with control mice (21.5 ± 2.4 fmol/mg/protein) 2 days after fear conditioning (Figure 4a). The increase was even more pronounced 8 days after fear conditioning (69.4 ± 5.3 fmol/mg protein in fear conditioned mice and 23.3 ± 3.6 fmol/mg in control mice, $n = 9$; $p < 0.0001$) (Figure 4b). In spite of this increased density, the immunohistochemical detection of A_{2A}Rs in amygdala sections was still near background (data not shown). A_{2A}R density also increased in other brain regions involved in the encoding of emotional traits, such as the hippocampus ($n = 9$) and ventral striatum ($n = 8$), whereas there was no alteration of A_{2A}R density in regions not directly implicated in encoding fear memories such as the dorsal striatum ($n = 8$) (Figures 4a and b).

We next tested the receptor specificity of these changes by determining whether fear conditioning altered A_1R density in different brain regions. As shown in Figure 4c, 2 days after fear acquisition, there was no modification of A_1R density in amygdala membranes of fear conditioned compared with control mice ($n = 5$); in contrast, there was an increased density of A_1Rs in the hippocampus ($n = 4$, $p < 0.05$) and in the ventral striatum ($n = 4$, $p < 0.05$), and no alteration in the dorsal striatum ($n = 4$) (Figure 4c). These alterations in A_1Rs were transient: 8 days after fear conditioning, there was a decreased density of A_1Rs in the hippocampus ($n = 5$, $p < 0.05$) and a tendency for a decrease in the amygdala ($n = 5$, $p = 0.082$) and in the ventral striatum ($n = 5$, $p = 0.061$) and no alterations in the dorsal striatum ($n = 5$) (Figure 4d).

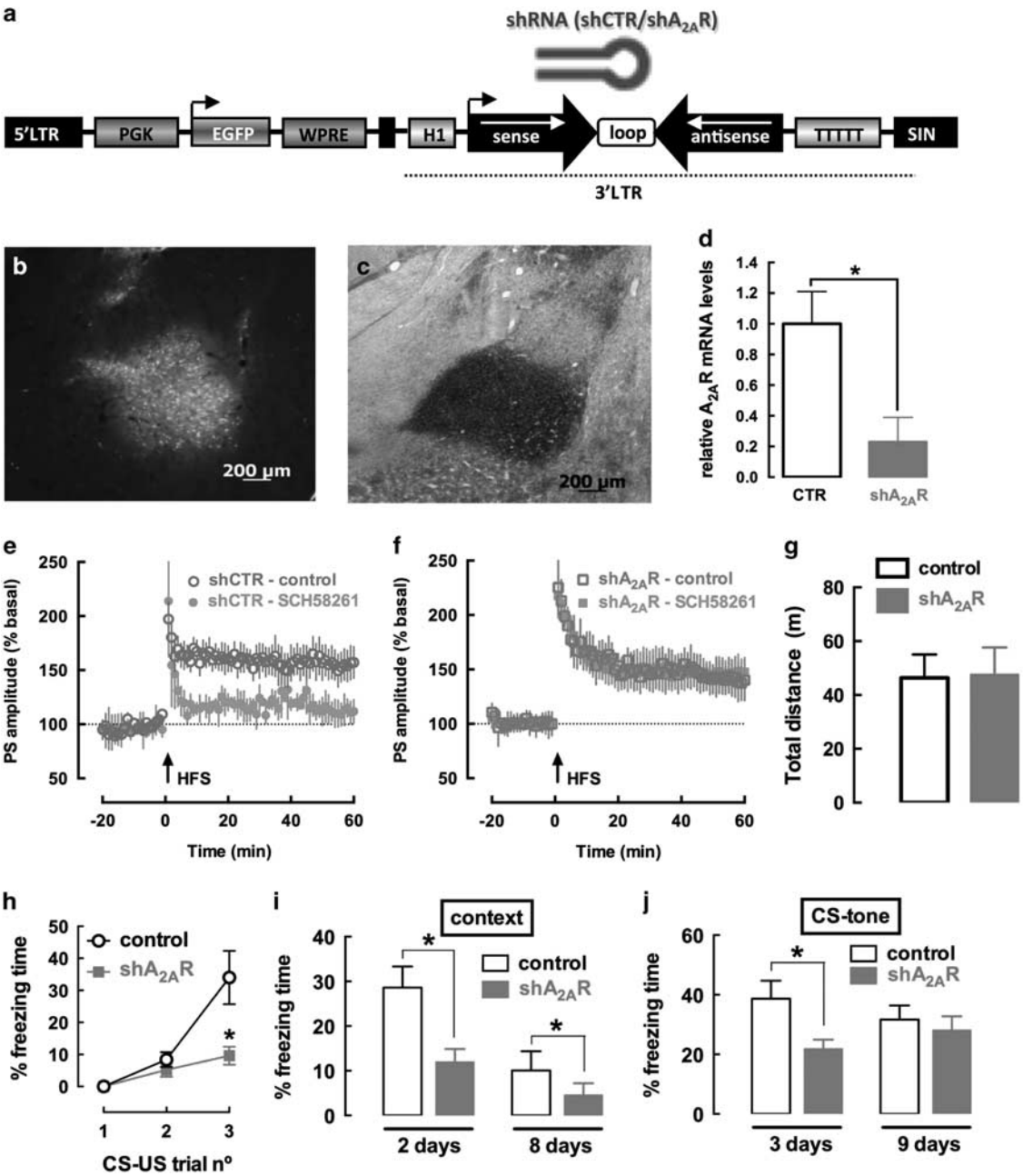


Figure 3 Lentivectors expressing a short hairpin RNA (shRNA) targeting adenosine A_{2A} receptors (A_{2A}Rs) effectively downregulate A_{2A}Rs and their bilateral injection in the basolateral complex of the amygdala decreases conditioned fear acquisition and expression. Lentivector constructs containing a sequence to neutralize A_{2A}Rs ($shA_{2A}R$) together with enhanced green fluorescent protein (EGFP) reporter (a) effectively transduced the basolateral complex, as gauged by the superimposition of EGFP labeling (b) with acetylcholinesterase staining (c), a marker of the basolateral complex (scale bar: 200 µm). When analyzed 4 weeks after $shA_{2A}R$ transduction in the amygdala (71.0 ± 8.8% of area transfected), there was a near 70% decrease of $A_{2A}R$ mRNA levels in the amygdala (d). The data are mean ± SEM of three of four mice; $^*p < 0.05$ with a Student's *t*-test. $shA_{2A}R$ caused a nonsignificant reduction ($p = 0.09$, $n = 3$, unpaired Student's *t*-test) of the number of glutamatergic nerve terminals (vGluT1 (vesicular glutamate transporter type 1)-immunopositive nerve terminals) endowed with A_{2A}Rs in the basolateral amygdala (BLA) (28.96 ± 1.20%) compared with control mice (38.32 ± 4.12%), but this only informs on the number of glutamatergic terminals endowed with A_{2A}Rs rather than on the amount of A_{2A}Rs in each terminal. The functional efficiency of $shA_{2A}R$ in the amygdala was confirmed by the elimination of the impact of SCH58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo-[1,5-c]pyrimidine; 50 nM) on long-term potentiation (LTP) amplitude in slices collected 3 weeks after the injection of $shA_{2A}R$ in the basolateral complex of the amygdala (f), whereas SCH58261 decreased LTP amplitude in slices from animals transfected with lentivectors lacking the silencing shRNA sequence to neutralize A_{2A}Rs (shCTR) (e). The data are mean ± SEM of four mice per group. (g) $shA_{2A}R$ and control mice displayed similar spontaneous locomotion in the open field. (h) The acquisition of a freezing response (time freezing during the 8 min test) to three repeated presentations of a 30-s tone conditioned stimulus (CS) paired with an unconditioned stimulus (US, 2-s foot-shock) 4 weeks after bilateral injection of $shA_{2A}R$ in the basolateral complex (filled symbols) showed a lower increase of freezing with each successive CS–US paired trial compared with mice treated with a lentivector without the shRNA silencing sequences to neutralize A_{2A}Rs (control, open symbols). (i) Context testing 1 or 7 days after fear acquisition showed a lower time of freezing in $shA_{2A}R$-treated mice compared with sh-control, which displayed a behavior superimposable to that of naïve mice. (j) Tone-induced freezing was also lower in $shA_{2A}R$-treated mice compared with sh-control when tested 2 days, but not 8 days, after fear conditioning. Data are mean ± SEM of five mice per group. $^*p < 0.05$ compared with the respective control (open symbols), two-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

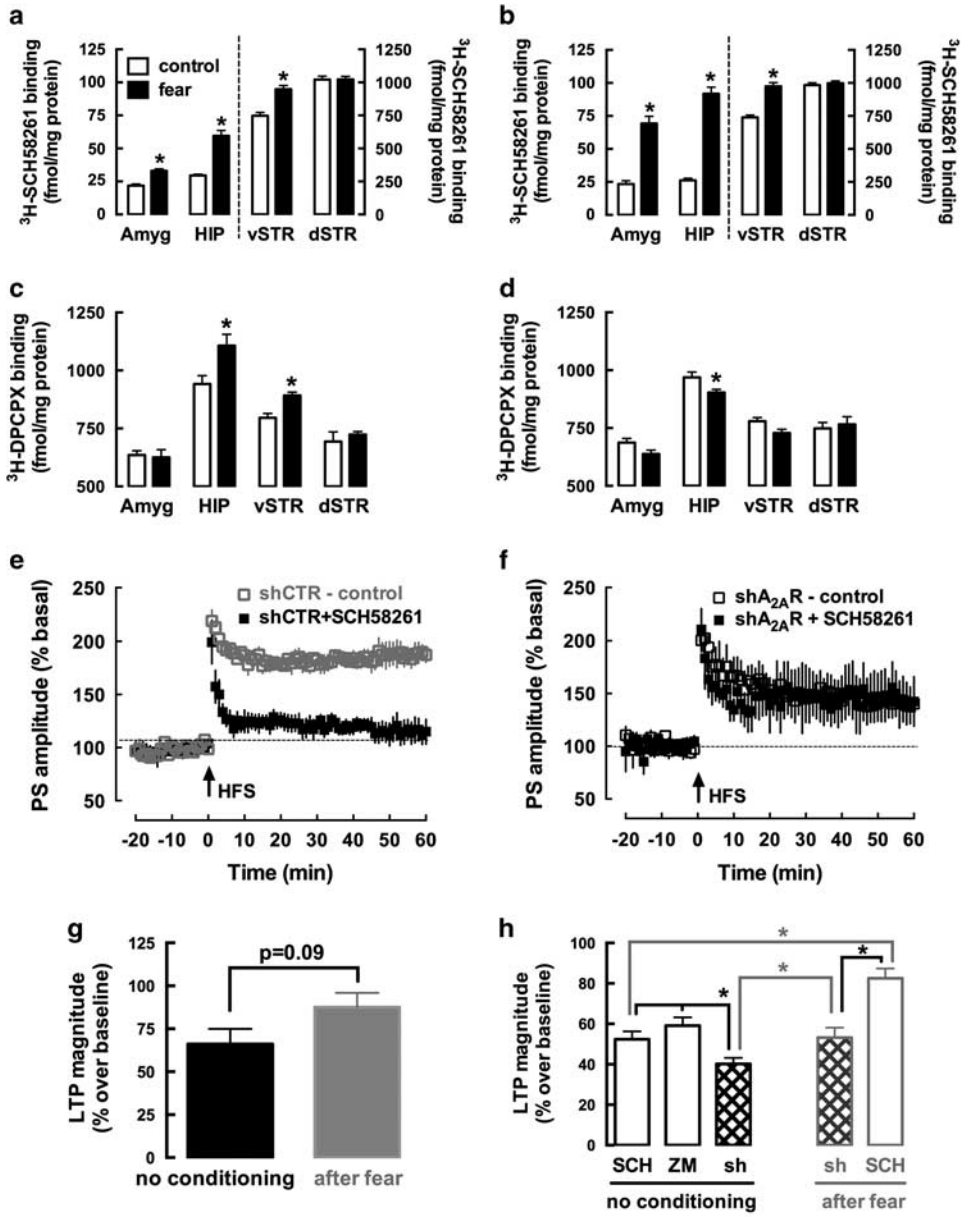


Figure 4 Conditioned fear triggers an increased density and a gain of function of adenosine A_{2A} receptors (A_{2A}Rs) in the amygdala. The comparison of the binding density of the selective A_{2A}R antagonist ^{3}H-SCH58261 (^{3}H-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo-[1,5-c]pyrimidine, 3 nM) to membranes prepared from control mice (open bars) and from fear conditioned mice 2 days (a) or 8 days (b) after fear conditioning (filled bars) revealed an increased A_{2A}R density in the amygdala (Amyg), hippocampus (HIP), and ventral striatum (vSTR), but not in the dorsal striatum (dSTR), of fear-stressed mice. This fear stress-induced upregulation seems selective for A_{2A}Rs as there was no similar modification of A_1R density, as evaluated by the binding density of the selective A_1R antagonist ^{3}H-DPCPX (^{3}H-1,3-dipropyl-8-cyclopenthylxanthine; 6 nM) in membranes from fear-stressed mice 2 days (c) or 8 days (d) after fear conditioning (filled bars) compared with control mice (open bars). Data are mean ± SEM of nine mice per group for analysis of A_{2A}R density and $n = 4$–5 mice per group for the analysis of A_1R density. *$p < 0.05$ compared with control (open symbols), unpaired Student's *t*-test. (e) The blockade of A_{2A}Rs with SCH58261 (50 nM) still effectively decreased long-term potentiation (LTP) amplitude recorded extracellularly in the lateral amygdala triggered by a high-frequency stimulation (HFS) train in external capsula of slices collected 8 days after fear conditioning; (f) such an effect that was not observed in slices collected 8 days after fear-stressed mice that were injected bilaterally in the amygdala 3 weeks before with lentivectors expressing a short hairpin RNA (shRNA) to neutralize A_{2A}Rs (shA_{2A}R). Data are mean ± SEM of four to five mice per group, unpaired Student's *t*-test. (g) Comparison of LTP amplitude before and 8 days after induction of fear conditioning. (h) Comparison of the impact on LTP amplitude of the different manipulations of A_{2A}Rs before and 8 days after induction of fear conditioning.

To test if this selective upregulation of A_{2A}R in the amygdala was associated with a modified functioning of A_{2A}Rs, we tested the ability of A_{2A}Rs to control LTP in amygdala slices collected 8 days after fear conditioning. As shown in Figure 4e, SCH58261 decreased ($p < 0.0001$) LTP amplitude (115.3 ± 10.5%, $n = 5$) in slices from fear conditioned mice, whereas LTP amplitude was 187.6 ± 8.3% ($n = 5$) in the absence of SCH58261. A comparison across different experimental groups suggested a tendency ($p = 0.09$; Figure 4g) for a larger LTP amplitude in slices collected

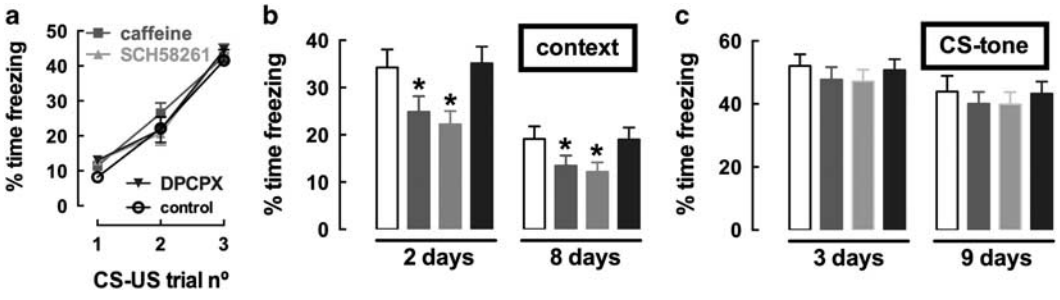
8 days after fear conditioning (187.6 ± 8.3%, $n = 5$) compared with slices from control mice not subject to fear conditioning (166.1 ± 8.8%, $n = 11$), whereas the extent of LTP inhibition upon $A_{2A}R$ blockade in slices from fear conditioned mice (82.5 ± 4.9%, $n = 5$) was larger ($p < 0.05$) compared with that observed in slices from naïve mice (52.4 ± 3.9%, $n = 6$; Figure 4h). This is in agreement with the proposed involvement of synaptic plastic changes in amygdala circuits to encode fear memory (Blair *et al*, 2001; Goosens and Maren, 2001; Johansen *et al*, 2011) and further documents a gain of function of $A_{2A}Rs$ to control LTP in the amygdala of fear conditioned mice.

$A_{2A}R$, but not A_1R, Blockade Impairs Long-Term Fear Memory Formation

In the last experiment, we sought to determine whether the global pharmacological blockade of $A_{2A}Rs$ controlled the acquisition of fear memory and if this was mimicked by caffeine (a nonselective adenosine receptor antagonist that is the most widely consumed psychoactive drug), but not by A_1R antagonists. Mice received daily injections either of caffeine (5 mg/kg per day), SCH58261 (0.1 mg/kg per day; $A_{2A}R$ antagonist), or DPCPX (0.5 mg/kg per day; A_1R antagonist), prior and throughout auditory fear conditioning. During fear conditioning (Figure 5a), all groups displayed increased freezing with successive CS–US pairings ($F_{2,30} = 38.9$, $p < 0.0001$). The acquisition of fear was similar across all treatments (Figure 5a). This suggests that this subchronic manipulation of A_1Rs and/or $A_{2A}Rs$ did not affect shock responsiveness, perception, or formation of the CS–US association. Additionally, spontaneous locomotion and nociceptive behavior ($n = 8$–10 *vs* saline-treated mice, $n = 11$) (Supplementary Figure 1) were not altered by our pharmacological manipulations.

Re-exposure of saline-treated mice to the conditioning context one day after conditioning induced freezing during 35.68 ± 3.19% ($n = 11$) of the 8 min exposure (Figure 5b); this was attenuated by caffeine (23.79 ± 3.04% freezing, $F_{1,152} = 28.43$, $p < 0.0001$) and SCH58261 (22.46 ± 2.59% freezing, $F_{1,152} = 39.04$, $p < 0.0001$), but not by DPCPX (34.92 ± 3.02% freezing, $F_{1,136} = 0.12$, $p = 0.73$). A similar pattern was observed when mice were re-exposed to the same context 7 days after conditioning (day 8) (Figure 5b): control mice froze during 18.43 ± 2.09% of the 8 min exposure ($n = 11$), which was decreased by caffeine (13.02 ± 1.81% freezing, $F_{1,152} = 11.29$, $p < 0.001$) and by SCH58261 (12.26 ± 1.46% freezing, $F_{1,152} = 16.11$, $p < 0.0001$), but not by DPCPX (19.07 ± 1.78% freezing, $F_{1,136} = 0.14$, $p = 0.71$).

As shown in Figure 5c, when mice were placed in a novel context 2 days after fear conditioning (day 3), control mice increased their freezing upon presentation of the CS (10.73 ± 1.36% before CS to 66.65 ± 2.66% after CS, $F_{7,77} = 11.03$, $p < 0.0001$); this was not modified by caffeine ($F_{1,152} = 1.77$, $p = 0.19$) or DPCPX ($F_{1,136} = 0.0008$, $p = 0.98$), but was decreased by SCH58261 ($F_{1,152} = 5.61$, $p < 0.05$). A similar pattern was observed when mice were exposed to the tone in a novel context 8 days after fear conditioning (day 9) (Figure 5c): control mice froze on presentation of CS (from 10.18 ± 1.33 to 63.37 ± 2.72%, $F_{7,77} = 11.03$, $p < 0.0001$) and this was decreased by caffeine ($F_{1,152} = 4.78$, $p < 0.05$) and by



d

	pharmacol [1] inhibition	amyg-sh [1]	fbKO [2]	stKO [2]	hip-sh [2]
acquisition	0	⇓	0	0	0
context fear	⇓	⇓	0	⇑	⇓
tone fear	0	⇓	⇓	⇑	0

Figure 5 Caffeine and selective adenosine A_{2A} receptor ($A_{2A}R$), but not A_1R, antagonists attenuate the expression of contextual fear. The acquisition of a freezing response (% freezing in the 8 min of test) to three repeated presentations of a 30-s tone conditioned stimulus (CS) paired with an unconditioned stimulus (US, 2-s foot-shock) are shown in (a) in control (vehicle-treated) mice (black open circles) and in mice treated intraperitoneally either with caffeine (5 mg/kg per day; squares) or the $A_{2A}R$-selective antagonist SCH58261 (amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo-[1,5-c]pyrimidine, 0.05 mg/kg per day; triangles) or the A_1R-selective antagonist DPCPX (1,3-dipropyl-8-cyclopenthylxanthine, 0.1 mg/kg per day, inverted triangles). All mice showed a comparable increase in freezing with each successive CS–US paired trial. (b) Freezing responses to the conditioning context in the absence of the CS were recorded 1 or 7 days later, showing that caffeine and SCH58261, but not DPCPX, treatments decreased context freezing. In contrast, tone CS testing, carried out in a different context, and evaluated either 2 or 8 days after fear conditioning (c) showed that none of the drug treatments significantly affected the percent time of freezing. Data are mean ± SEM of 8–11 mice per group. *$p < 0.05$ compared with control (vehicle-treated), one-way analysis of variance (ANOVA) followed by Turkey's *post hoc* test. (d) Summary table of the impact of the different manipulation of $A_{2A}Rs$ in different brain regions on the acquisition and expression of conditioned fear. (1) This study; (2) Wei *et al* (2014). A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

SCH58261 ($F_{1,152} = 4.38$, $p < 0.05$), but not by DPCPX ($F_{1,136} = 0.06$, $p = 0.81$).

These data indicate that caffeine and selective $A_{2A}R$ inhibition decreased the expression of contextual fear memory, whereas A_1R blockade was devoid of effects. This ability of $A_{2A}Rs$ to control fear memory was further tested by comparing wild type (WT) and global $A_{2A}R$-KO mice. As shown in Supplementary Figure 2, $A_{2A}R$-KO and WT mice displayed a similar acquisition of fear ($F_{1,48} = 0.39$, $p = 0.53$ for genotype and $F_{2,48} = 19.15$, $p < 0.0001$ for trials). When tested for contextual fear 1 or 7 days after conditioning, $A_{2A}R$-KO mice froze less than WT mice ($F_{1,128} = 13.99$, $p < 0.0005$ at day 2; $F_{1,128} = 4.88$, $p < 0.05$ at day 8); in contrast, when probed in a novel context for auditory fear memory, there was no significant difference between genotypes either 2 days ($F_{1,128} = 0.004$, $p = 0.95$) or 8 days ($F_{1,128} = 0.11$, $p = 0.74$) after fear conditioning (Supplementary Figure 2).

DISCUSSION

The present study identifies $A_{2A}Rs$ as novel key regulators of synaptic plasticity in the amygdala and of fear memory. Thus, the global pharmacological inhibition of $A_{2A}Rs$ and the downregulation of $A_{2A}Rs$ selectively in the basolateral complex of the amygdala impaired fear memory, in accordance with the ability of $A_{2A}R$ blockade to selectively dampen the amplitude of long-term potentiation in this region.

The expression of contextual fear was decreased by caffeine consumption and by the genetic and pharmacological blockade of $A_{2A}Rs$, but was unaffected upon selective blockade of inhibitory A_1Rs; given that caffeine at non-toxic doses mostly targets A_1Rs and $A_{2A}Rs$ (Fredholm *et al*, 2005), this indicates that the impact of caffeine consumption on contextual fear memory was likely mediated by the selective antagonism of $A_{2A}Rs$, as previously proposed for other behavioral responses (Cunha and Agostinho, 2010). This effect contrasts with the previously reported disruptive effects of acutely administered higher doses of caffeine (Corodimas *et al*, 2000), further highlighting the care to use 'physiological' doses of caffeine and to use schedules of administrations that mimic caffeine consumption in humans. In parallel, we also observed that $A_{2A}Rs$ controlled synaptic plasticity processes in the lateral amygdala, the purported neurophysiological basis of conditioned fear (Johansen *et al*, 2011; Goosens and Maren, 2001; Blair *et al*, 2001). Thus, different $A_{2A}R$ antagonists attenuated LTP amplitude in amygdala slices, an effect inexistent upon treatment with $shA_{2A}R$ as well as in global $A_{2A}R$-KO mice; this also testifies that, although we did not directly quantify the reduction of $A_{2A}Rs$ in the amygdala after $shA_{2A}R$ treatment, the achieved downregulation of amygdala $A_{2A}Rs$ with $shA_{2A}R$ was sufficient to eliminate $A_{2A}R$-mediated responses. Notably, $A_{2A}Rs$ were selectively engaged to control long-term plastic processes and were devoid of effects on the control of basal synaptic transmission or of short-term plasticity, as occurred in hippocampal (Rebola *et al*, 2008; Costenla *et al*, 2011) or striatal synapses (d'Alcantara *et al*, 2001; Flajolet *et al*, 2008). This impact on synaptic plasticity is in agreement with the enrichment of $A_{2A}Rs$ in synapses within the amygdala and in particular with the localization of $A_{2A}Rs$ in glutamatergic synapses, as occurs in other limbic regions such as the hippocampus (Rebola *et al*, 2005a, b; Costenla *et al*, 2011). However, our data does not allow distinguishing between possible pre- and postsynaptic effects of $A_{2A}Rs$ (Rau *et al*, 2015) to control LTP, which will need additional analysis using whole-cell patch-clamp recordings. Similarly, additional studies are required to test if ATP-derived adenosine is also the source of adenosine-activating $A_{2A}Rs$ and if ATP is released during induction of synaptic plasticity in amygdala synapses, which is currently unknown. This parallel ability of $A_{2A}Rs$ to control amygdala synaptic plasticity and fear-related responses also raises the question of disentangling if $A_{2A}Rs$ are continuously affecting an abnormal functioning of amygdala circuits, as suggested by the sustained upregulation of $A_{2A}Rs$ in the amygdala after fear acquisition, or if instead $A_{2A}Rs$ are critically required for a state-dependent shift on exposure to fear, as suggested by the ability of $A_{2A}Rs$ to control the emotional status of rodents (Wei *et al*, 2014; Kaster *et al*, 2015).

This key role of amygdala $A_{2A}Rs$ to control fear memory was directly confirmed by our observation that the selective bilateral downregulation of $A_{2A}Rs$ in the amygdala was sufficient to decrease fear memory. Importantly, we defined that this $A_{2A}R$ downregulation occurred in neurons (see Viana da Silva *et al*, 2016) and abolished the impact of $A_{2A}Rs$ on amygdala LTP, but we could not disentangle the relative effect of $shA_{2A}R$ on pre- and postsynaptic $A_{2A}Rs$. However, $shA_{2A}R$-treated mice displayed a decreased acquisition of conditioned fear and a decreased expression of both contextual and cued fear, whereas the global blockade of $A_{2A}Rs$ selectively dampened contextual fear without effects on acquisition and cued fear. This was not due to an effect of caffeine or of selective $A_{2A}R$ antagonists on nociception, as we used a dose of SCH58261 one order of magnitude smaller than the minimum dose previously shown to have no effect on nociception (Bastia *et al*, 2002). Instead, the different impact of the global $A_{2A}R$ blockade compared with the blockade of $A_{2A}Rs$ selectively in the amygdala indicates that the impact of $A_{2A}Rs$ on fear memory is unlikely to be restricted to the amygdala, in accordance with the previously reported ability of hippocampal $A_{2A}Rs$ to interfere with contextual fear and the opposite effect of amygdala and striatal $A_{2A}Rs$ on the control of the expression of fear memory (Wei *et al*, 2014), as summarized in Figure 5d. In fact, the acquisition and recall of conditioned fear also involves other limbic and neocortical areas in partially redundant circuits (Orsini and Maren, 2012). Similarly, it is possible that the selective deletion of $A_{2A}Rs$ in the amygdala might bolster the impact of otherwise less relevant $A_{2A}Rs$ in other brain regions, as we have previously observed to occur for the recruitment of striatal DARPP-32 (Shen *et al*, 2013), behavioral sensitization (Shen *et al*, 2008), or emotional responses (Wei *et al*, 2014) using cell-type-selective genetic eliminations of $A_{2A}Rs$. In fact, several studies have dissected the involvement of different brain regions in the processing of contextual and cued fear memory (Orsini and Maren, 2012), albeit the expression of both forms of contextual fear mostly depend on amygdala circuits (Goosens and Maren, 2001). This is heralded by the differential impact on cued and contextual fear memory upon manipulation of different molecular targets (eg, Sui *et al*, 2006; Burghart and Bauer,

2013) or lesions/inactivation (eg, Duvarci *et al*, 2009; Baldi *et al*, 2013) in different brain regions. This apparent different involvement of A_{2A}Rs in the amygdala and in other brain regions in the acquisition and expression of contextual fear, which still remains to be detailed, does not undermine the key impact of A_{2A}R blockade in long-term contextual fear, a conclusion of particular importance in view of the prominent role of contextualization in behavioral flexibility and psychopathology (reviewed in Maren *et al*, 2013).

The relevance of this novel A_{2A}R-mediated control of conditional fear is bolstered by the reported observations that the implementation of fear memory traits is accompanied by an upregulation of A_{2A}Rs in the amygdala together with a gain of function of A_{2A}Rs controlling amygdala LTP. A_{2A}R upregulation was also detected in other brain regions involved in the processing of emotional information, such as the hippocampus and ventral striatum, and is in agreement with the previous observation that stressful events upregulate A_{2A}Rs (Fredholm *et al*, 2005; Cunha and Agostinho, 2010). Furthermore, A_{2A}Rs displayed a gain of function in the control of amygdala LTP after fear stress and the blockade of A_{2A}Rs decreased excessive plasticity in the amygdala, as it was previously found to occur in the hippocampus (Costenla *et al*, 2011) and in the striatum (Li *et al*, 2015b). This makes A_{2A}Rs attractive targets to manage conditions associated with abnormal fear expression, namely upon post-traumatic stress disorders. This is supported by the association between A_{2A}R polymorphisms with phobia and panic attacks (Deckert *et al*, 1998; Hamilton *et al*, 2004) and by the observed inverse correlation between caffeine intake and the incidence of depression (Lucas *et al*, 2011) and suicides (Lucas *et al*, 2013).

In conclusion, the present study provides combined pharmacological and genetic evidence that A_{2A}R blockade decreases fear memory. The study also identifies the presence of A_{2A}Rs in glutamatergic terminals in the amygdala, where they selectively control synaptic plasticity processes that are considered the neurophysiological basis of conditional fear memory. Finally, the observed increased density of amygdala A_{2A}Rs and the gain of function of A_{2A}Rs to control synaptic plasticity in the lateral amygdala after fear conditioning prompt the provocative novel hypothesis that A_{2A}Rs may have a key role in the acquisition and preservation of contextual fear memories. This paves the way to consider A_{2A}R antagonists as novel candidate drugs to manage psychiatric conditions associated with excessive expression of aversive memories such as post-traumatic stress disorders.

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RAC is a scientific consultant for the Institute for Scientific Information on Coffee. The other authors declare no conflict of interest.

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REFERENCES

Alves S, Régulier E, Nascimento-Ferreira I, Hassig R, Dufour N, Koeppen A *et al* (2008). Striatal and nigral pathology in a lentiviral rat model of Machado–Joseph disease. *Hum Mol Genet* **17**: 2071–2083.

Baldi E, Liuzzo A, Bucherelli C (2013). Fimbria–fornix and entorhinal cortex differential contribution to contextual and cued fear conditioning consolidation in rats. *Physiol Behav* **114–115**: 42–48.

Bastia E, Varani K, Monopoli A, Bertorelli R (2002). Effects of A_1 and A_{2A} adenosine receptor ligands in mouse acute models of pain. *Neurosci Lett* **328**: 241–244.

Batalha VL, Pego JM, Fontinha BM, Costenla AR, Valadas JS, Baqi Y *et al* (2013). Adenosine A_{2A} receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation. *Mol Psychiatry* **18**: 320–331.

Berdel B, Moryś J, Maciejewska B, Narkiewicz O (1996). Acetylcholinesterase activity as a marker of maturation of the basolateral complex of the amygdaloid body in the rat. *Int J Dev Neurosci* **14**: 543–549.

Blair HT, Schafe GE, Bauer EP, Rodrigues SM, LeDoux JE (2001). Synaptic plasticity in the lateral amygdala: a cellular hypothesis of fear conditioning. *Learn Mem* **8**: 229–242.

Burghardt NS, Bauer EP (2013). Acute and chronic effects of selective serotonin reuptake inhibitor treatment on fear conditioning: implications for underlying fear circuits. *Neuroscience* **247**: 253–272.

Chen JF (2014). Adenosine receptor control of cognition in normal and disease. *Int Rev Neurobiol* **119**: 257–307.

Corodimas KP, Pruitt JC, Stieg JM (2000). Acute exposure to caffeine selectively disrupts context conditioning in rats. *Psychopharmacology* **152**: 376–382.

Costenla AR, Diógenes MJ, Canas PM, Rodrigues RJ, Nogueira C, Maroco J *et al* (2011). Enhanced role of adenosine A_{2A} receptors in the modulation of LTP in the rat hippocampus upon ageing. *Eur J Neurosci* **34**: 12–21.

Cunha RA (2008). Different cellular sources and different roles of adenosine: A_1 receptor-mediated inhibition through astrocytic-driven volume transmission and synapse-restricted A_{2A} receptor-mediated facilitation of plasticity. *Neurochem Int* **52**: 65–72.

Cunha RA, Agostinho PM (2010). Chronic caffeine consumption prevents memory disturbance in different animal models of memory decline. *J Alzheimer Dis* **20**(Suppl 1): 95–116.

D'Alcantara P, Ledent C, Swillens S, Schiffmann SN (2001). Inactivation of adenosine A_{2A} receptor impairs long term potentiation in the accumbens nucleus without altering basal synaptic transmission. *Neuroscience* **107**: 455–464.

Deckert J, Nöthen MM, Franke P, Delmo C, Fritze J, Knapp M *et al* (1998). Systematic mutation screening and association study of the A_1 and A_{2A} adenosine receptor genes in panic disorder suggest a contribution of the A_{2a} gene to the development of disease. *Mol Psychiatry* **3**: 81–85.

Duvarci S, Bauer EP, Paré D (2009). The bed nucleus of the stria terminalis mediates inter-individual variations in anxiety and fear. *J Neurosci* **29**: 10357–10361.

Flajolet M, Wang Z, Futter M, Shen W, Nuangchamnong N, Bendor J *et al* (2008). FGF acts as a co-transmitter through adenosine A_{2A} receptor to regulate synaptic plasticity. *Nat Neurosci* **11**: 1402–1409.

Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM (2005). Adenosine and brain function. *Int Rev Neurobiol* **63**: 191–270.

Goosens KA, Holt W, Maren S (2000). A role for amygdaloid PKA and PKC in the acquisition of long-term conditional fear memories in rats. *Behav Brain Res* **114**: 145–152.

Goosens KA, Maren S (2001). Contextual and auditory fear conditioning are mediated by the lateral, basal and central amygdaloid nuclei in rats. *Learn Mem* **8**: 148–155.

Hamilton SP, Slager SL, De Leon AB, Heiman GA, Klein DF, Hodge SE *et al* (2004). Evidence for genetic linkage between a polymorphism in the adenosine 2A receptor and panic disorder. *Neuropsychopharmacology* **29**: 558–565.

Johansen JP, Cain CK, Ostroff LE, LeDoux JE (2011). Molecular mechanisms of fear learning and memory. *Cell* **147**: 509–524.

Kaster MP, Machado NJ, Silva HB, Nunes A, Ardais AP, Santana M *et al* (2015). Caffeine acts through neuronal adenosine A_{2A} receptors to prevent mood and memory dysfunction triggered by chronic stress. *Proc Natl Acad Sci USA* **112**: 7833–7838.

Laurent C, Burnouf S, Ferry B, Batalha VL, Coelho JE, Baqi Y *et al* (2016). A_{2A} adenosine receptor deletion is protective in a mouse model of Tauopathy. *Mol Psychiatry* **21**: 97–107.

Le Bars D, Gozariu M, Cadden SW (2001). Animal models of nociception. *Pharmacol Rev* **53**: 597–652.

Li P, Rial D, Canas PM, Yoo JH, Li W, Zhou X *et al* (2015a). Optogenetic activation of intracellular adenosine A_{2A} receptor signaling in the hippocampus is sufficient to trigger CREB phosphorylation and impair memory. *Mol Psychiatry* **20**: 1339–1349.

Li W, Silva HB, Real J, Wang YM, Rial D, Li P *et al* (2015b). Inactivation of adenosine A_{2A} receptors reverses working memory deficits at early stages of Huntington's disease models. *Neurobiol Dis* **79**: 70–80.

Lucas M, Mirzaei F, Pan A, Okereke OI, Willett WC, O'Reilly ÉJ *et al* (2011). Coffee, caffeine, and risk of depression among women. *Arch Intern Med* **171**: 1571–1578.

Lucas M, O'Reilly EJ, Pan A, Mirzaei F, Willett WC, Okereke OI *et al* (2013). Coffee, caffeine, and risk of completed suicide: results from three prospective cohorts of American adults. *World J Biol Psychiatry* **15**: 377–386.

Lundberg C, Björklund T, Carlsson T, Jakobsson J, Hantraye P, Déglon N *et al* (2008). Applications of lentiviral vectors for biology and gene therapy of neurological disorders. *Curr Gene Ther* **8**: 461–473.

Mahan AL, Ressler KJ (2012). Fear conditioning, synaptic plasticity and the amygdala: implications for posttraumatic stress disorder. *Trends Neurosci* **35**: 24–35.

Maren S, Phan KL, Liberzon I (2013). The contextual brain: implications for fear conditioning, extinction and psychopathology. *Nat Rev Neurosci* **14**: 417–428.

Nakamura M, Nakakimura K, Matsumoto M, Sakabe T (2002). Rapid tolerance to focal cerebral ischemia in rats is attenuated by adenosine A_1 receptor antagonist. *J Cereb Blood Flow Metab* **22**: 161–170.

Orr AG, Hsiao EC, Wang MM, Ho K, Kim DH, Wang X *et al* (2015). Astrocytic adenosine receptor A_{2A} and G_s-coupled signaling regulate memory. *Nat Neurosci* **18**: 423–434.

Orsini CA, Maren S (2012). Neural and cellular mechanisms of fear and extinction memory formation. *Neurosci Biobehav Rev* **36**: 1773–1802.

Pagnussat N, Almeida AS, Marques DM, Nunes F, Chenet GC, Botton PH *et al* (2015). Adenosine A_{2A} receptors are necessary and sufficient to trigger memory impairment in adult mice. *Br J Pharmacol* **172**: 3831–3845.

Prediger RD, Batista LC, Takahashi RN (2005). Caffeine reverses age-related deficits in olfactory discrimination and social recognition memory in rats. Involvement of adenosine A_1 and A_{2A} receptors. *Neurobiol Aging* **26**: 957–964.

Rau AR, Ariwodola OJ, Weiner JL (2015). Postsynaptic adenosine A_{2A} receptors modulate intrinsic excitability of pyramidal cells in the rat basolateral amygdala. *Int J Neuropsychopharmacol* **18**: pyv017.

Rebola N, Canas PM, Oliveira CR, Cunha RA (2005a). Different synaptic and subsynaptic localization of adenosine A_{2A} receptors in the hippocampus and striatum of the rat. *Neuroscience* **132**: 893–903.

Rebola N, Lujan R, Cunha RA, Mulle C (2008). Adenosine A_{2A} receptors are essential for long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses. *Neuron* **57**: 121–134.

Rebola N, Rodrigues RJ, Lopes LV, Richardson PJ, Oliveira CR, Cunha RA (2005b). Adenosine A_1 and A_{2A} receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus. *Neuroscience* **133**: 79–83.

Shen HY, Canas PM, Garcia-Sanz P, Lan JQ, Boison D, Moratalla R *et al* (2013). Adenosine A_{2A} receptors in striatal glutamatergic terminals and GABAergic neurons oppositely modulate psychostimulant action and DARPP-32 phosphorylation. *PLoS One* **8**: e80902.

Shen HY, Coelho JE, Ohtsuka N, Canas PM, Day YJ, Huang QY *et al* (2008). A critical role of the adenosine A_{2A} receptor in extrastriatal neurons in modulating psychomotor activity as revealed by opposite phenotypes of striatum and forebrain A_{2A} receptor knock-outs. *J Neurosci* **28**: 2970–2975.

Sui L, Wang F, Liu F, Wang J, Li BM (2006). Dorsal hippocampal administration of triiodothyronine enhances long-term memory for trace cued and delay contextual fear conditioning in rats. *J Neuroendocrinol* **18**: 811–819.

Viana da Silva S, Haberl MG, Zhang P, Bethge P, Lemos C, Gonçalves N *et al* (2016). Early synaptic deficits in the APP/PS1 mouse model of Alzheimer's disease involve neuronal adenosine A2A receptors. *Nature Comm* **7**: 11915.

Wei CJ, Augusto E, Gomes CA, Singer P, Wang Y, Boison D *et al* (2014). Regulation of fear responses by striatal and extrastriatal adenosine A_{2A} receptors in forebrain. *Biol Psychiatry* **75**: 855–863.

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