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ORIGINAL ARTICLE

Adenosine A_{2A} receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation

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Maternal separation (MS) is an early life stress model that induces permanent changes in the central nervous system, impairing hippocampal long-term potentiation (LTP) and spatial working memory. There are compelling evidences for a role of hippocampal adenosine A_{2A} receptors in stress-induced modifications related to cognition, thus opening a potential window for therapeutic intervention. Here, we submitted rats to MS and evaluated the long-lasting molecular, electrophysiological and behavioral impairments in adulthood. We then assessed the therapeutic potential of KW6002, a blocker of A_{2A} receptors, in stress-impaired animals. We report that the blockade of A_{2A} receptors was efficient in reverting the behavior, electrophysiological and morphological impairments induced by MS. In addition, this effect is associated with restoration of the hypothalamic-pituitary-adrenal axis (HPA-axis) activity, as both the plasma corticosterone levels and hippocampal glucocorticoid receptor expression pattern returned to physiological-like status after the treatment. These results reveal the involvement of A_{2A} receptors in the stress-associated impairments and directly in the stress response system by showing that the dysfunction of the HPA-axis as well as the long-lasting synaptic and behavioral effects of MS can be reverted by targeting adenosine A_{2A} receptors. These findings provide a novel evidence for the use of adenosine A_{2A} receptor antagonists as potential therapy against psychopathologies.
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Keywords: adenosine A_{2A} receptors; corticosterone; hippocampus; HPA-axis; maternal separation; stress

Introduction

Exposure to stress has deleterious effects on brain structure and function, which could be manifested either immediately after stress,[1] as a long-term vulnerability to cognitive deficits[2] or even as an increased susceptibility to neuropsychiatric disorders, where stress has a major role.[3,4]

Mother–infant interaction is a key factor for brain maturation and disease susceptibility which in humans can manifest in cognitive and behavioral disorders later in life.[5,6] In rats, the daily separation of the litter from their mothers for 180 min each day during postnatal days (PNDs) 2–14 will result in an alteration of maternal behavior, namely with a significant reduction in licking/grooming duration.[7] During this period, the hippocampus, which is critically involved in long-term memory formation[8] and is also a primary target for stress hormones in the central nervous system,[1,9] goes through great development. The majority of hippocampal granule neurons develop and extend their axons between PND 1 and 21[10] and the peak period of neurogenesis and mossy fiber outgrowth overlaps with the stress hyporesponsive period (PND 4–14) in neonatal rats.[11] This will induce changes that persist throughout adult life at the level of gene expression, neurochemistry, electrophysiology proprieties and morphology[12,13] with behavioral and neuroendocrine signs of cognitive deficits and over-activation of the hypothalamic-pituitary-adrenal axis (HPA-axis) as adults.[7,14–16]

Adenosine receptors in the hippocampus are important modulators of synaptic transmission and neuronal excitability. Glutamatergic synaptic transmission in physiological conditions is controlled

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negatively by the dominant adenosine A_1 receptors, and positively to a lesser extent by A_{2A} receptors.[17] Interestingly, this pattern appears to be modified in the aged hippocampus, with a marked increase in the expression of A_{2A} receptors and a decrease in the expression of A_1 receptors.[18,19] These changes are accompanied by a strong direct facilitatory effect of A_{2A} receptors on the release of glutamate.[20] This is also observed in other situations associated to neuronal dysfunction, such as epilepsy, acute stress or animal models of Alzheimer's disease,[21] which suggests a deleterious contribution of A_{2A} receptors to these conditions. The blockade of A_{2A} receptors was proven beneficial against synaptic loss associated with acute stress in the hippocampus.[22] Interestingly, cognitive impairments also occur when excessive levels of corticosteroids are attained due to disease, or due to hypersecretion in response to a stressor.[23,24]

However, it is still unknown the extent to which A_{2A} receptors are involved in the long-term effects of early life stress. Here, we submitted rats to maternal separation (MS) and evaluated the long-lasting molecular, electrophysiological and behavioral impairments at adult age. We then assessed the therapeutic potential of blocking endogenous activation of A_{2A} receptors, by administering a selective antagonist, KW6002 (istradefylline), orally for 1 month to stress-impaired animals. We report that the blockade of A_{2A} receptors was efficient in reverting the long-lasting behavior, morphological and electrophysiological impairments induced by MS. We also show that this effect is associated with the re-establishment of the HPA-axis activity, as both the plasma corticosterone levels and hippocampal glucocorticoid receptor expression pattern returned to physiological-like status after the treatment.

Materials and methods

Animals

Pregnant Wistar rats were purchased (Harlan, Barcelona, Spain) in mid-gestation and were due in our animal facility. All animals were handled according to European Community guidelines and Portuguese law on animal care (1005/92). The animals that were killed by decapitation were anesthetized under halothane atmosphere.

MS protocol

The protocol used has been previously validated and described.[25] Wistar dams and their litters were assigned either to the control (CTR—non-separated) or to the MS groups as described before.[26] To exclude artifacts from genetic background, at PND 2, all the litters were collected together, gender assessed and the pups were randomly distributed to foster dams (gender proportion maintained). MS pups were removed from their cages as a group from PND 2 to 14, for 180 min, daily, at 9 am, and placed in an isolation cage in an adjacent room kept at $32.0 \pm 0.5\,^{\circ}C$. At the end of the separation period, pups were returned to their home-cage and rolled in the soiled home cage bedding before reuniting with the mother. CTR pups were not handled and were maintained in their home-cages until weaning. At day 21 the pup's gender was confirmed, males weaned and housed in groups of 5–8 animals per cage until use at adult age (8–14 weeks; according to diagram in Supplementary Figure S1).

Oral administration of the drug

KW6002 (istradefylline), a selective adenosine A_{2A} receptor antagonist[27] was orally administered diluted in the drinking water, being continuously available. The weight of the animals and the volume intake were assessed twice a week and the concentration of the solution was adjusted so that the drug intake was maintained at $3\,mg\,kg^{-1}$ per day. Animals were divided in four groups: CTR or MS, drinking vehicle (0.025% methylcellulose) and CTR KW or MS KW drinking KW6002 ($3\,mg\,kg^{-1}$ per day, 0.025% methylcellulose). The treatment started at 4–6 weeks old, and was prolonged for 1 month until sacrifice. The KW6002 administration was kept throughout the behavioral assessments.

Corticosterone quantification

Blood was extracted from the tail, in animals previously handled to minimize stress and without anesthesia, at two different time points, 8 am (nadir) and 8 pm (zenith). The plasma was isolated by centrifugation at 2000 g, 4 °C for 15 min and corticosterone quantified by radioimmunoassay using the rat corticosterone ^{3}H kit from MP Biomedicals, UK according to the manufacturer's protocol.

Behavioral assessments

CTR, MS, CTR KW, MS KW were first handled for 5 days before testing the behavior assays, that were performed in the following sequence: open-field (OF), Elevated plus maze (EPM) and Morris water maze (MWM):[28] Rats were given spatial acquisition training consisting of four trials/day for 4 consecutive days, as performed before.[29] On the 5th day a probe test was given in which the platform was removed and animals were allowed to swim freely for 60 s while recording the percentage of time spent on each quadrant. The latency to found the platform during acquisition and the percentage of time in the platform quadrant in probe test were used to evaluate hipppocampal-dependent memory. EPM: The maze is shaped like a plus sign and consists of two 'open' and two 'closed' arms, arranged perpendicularly, and elevated 50 cm above the floor. Each animal was placed at the center of the equipment, facing an open arm. Each test lasted 5 min and all testing sessions were performed between 10:00 am and 17:00 pm in a sound-attenuated room. The maze was cleaned with a 70% ethanol solution between each animal. The total time spent in the open arms and the total arms entries (number of entries in open + closed arms) were used as anxiety and locomotor measures.[30] Open field:

The animals were placed at the center of the arena (66×66 cm) and allowed to explore for 5 min. Changes in mean speed and path length of the subjects were continuously monitored by an automated tracking system (Smart 2.5, PanLab, Barcelona, Spain). The maze was cleaned with a 70% ethanol solution between each animal.

Histological procedures
The day after the last testing session, five rats from each experimental group were perfused transcardially with phosphate-buffered saline, under deep pentobarbital anesthesia. Brains were removed and split into two hemispheres, and processed either for stereology, or for Golgi-Cox staining according to the procedures previously described.[31,32] Briefly, for stereology the left hemispheres were included in glycolmethacrylate (Tecnovit 7100; Heraeus Kulzer, Werheim, Germany) and every other microtome-cut section (30 μm) was then collected on a gelatinized slide, stained with Giemsa, and mounted with Entellan New (Merck, Darmstadt, Germany). The shrinkage factor was calculated according to Madeira *et al*.[33] For 3D neuronal reconstructions, hemispheres were removed and immersed in Golgi-Cox solution (a 1:1 solution of 5% potassium dichromate and 5% mercuric chloride diluted 4:10 with 5% potassium chromate[34]) for 14 days; hemispheres were then transferred to a 30% sucrose solution (3 days), before being cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Linda-a-Velha, Portugal), fixed in Kodak Rapid Fix (prepared as manufacturer instructions), dehydrated through a graded series of ethanols, and cleared in xylene before being mounted and coverslipped. Slides were coded before morphometric analysis in both sets.

Region and layer boundaries
We analyzed the following regions of the hipocampal formation: the dentate gyrus (including polymorphic, granule cell layer and molecular layer), CA1 (strata oriens, pyramidale, radiatum and lacunosum-moleculare) and CA3 (strata oriens, pyramidale, lucidum and radiatum). The above-mentioned regions were outlined according to the atlas of Paxinos and Watson,[35] based on noticeable cytoarchitectural differences.[36]

Stereological procedures
Volume estimations were performed using StereoInvestigator software (MicroBrightField, Williston, VT, USA) and a camera (DXC390; Sony, Tokyo, Japan) attached to a motorized microscope (Axioplan 2; Zeiss, Oberkochen, Germany). Cavalieri's principle[37] was used to assess the volume of each region. Briefly, every 10th section was used and its cross-sectional area was estimated by point counting at a final magnification of $\times 112$. For this, we randomly superimposed onto each area a test point grid in which the interpoint distance, at tissue level, was as follows: (1) 150 μm for the three layers of the dentate gyrus, (2) 250 μm for the three layers of CA1 and CA3. The volume of the region of interest was calculated from the number of points that fell within its boundaries and the distance between the systematically sampled sections.

Dendritic tree analysis
Three-D reconstructions of representative Golgi-impregnated neurons from CA1 were made. The criteria used to select neurons for reconstruction were as follows: (i) full impregnation of the neurons along the entire length of the dendritic tree; (ii) dendrites without significant truncation of branches; (iii) relative isolation from neighboring impregnated neurons to avoid interference with the analysis; (iv) no morphological changes attributable to incomplete dendritic impregnation of Golgi–Cox stain. Golgi-impregnated pyramidal-like neurons of the CA1 region were readily identified by their characteristic pyramidal or piriform soma, spine-sparse primary dendrites and spine-dense secondary dendrites (Figure 2e for representative reconstructions). For each selected neuron, all branches of the dendritic tree and the location of all dendritic spines were reconstructed at $\times 600$ magnification, using a motorized microscope (Carl Zeiss Axioplan 2, Hamburg, Germany, with oil-objectives), attached to a camera (DXC-390, Sony, Tokyo, Japan) and Neurolucida software (Microbrightfield). Three-D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). In each hemisphere, 10 CA1 pyramidal neurons were reconstructed; as a result in this study we have analyzed 200 neurons. Several aspects of dendritic morphology were examined. To assess overall changes, total dendritic length, number of ramifications and number of dendrites were compared between groups. Sholl analysis was performed to assess changes in the ramification pattern.

Electrophysiological recordings
After decapitation the brain was rapidly removed and the hippocampi were dissected free in ice-cold Krebs solution composed of (mM): NaCl 124; KCl 3; NaH2PO4 1.25; NaHCO3 26; MgSO4 1; CaCl2 2; and glucose 10, previously gassed with 95% O_2 and 5% CO_2, pH 7.4. 400 μM slices were obtained with a McEwen tissue shopper and field excitatory postsynaptic potentials (fEPSPs) were recorded as previously[29] in *stratum radiatum* of the CA1 area. Input–output (I/O) curves and long-term potentiation (LTP, 100 Hz, 1 s, 100 pulses induced at 0.5 mV/ms; $<50\%$ max) were recorded as previously. The second hippocampus was rapidly frozen in liquid nitrogen for further analysis.

Tissue processing
Samples were homogenized either in radio immunoprecipitation-assay buffer (50 mM Tris, 1 mM EDTA,

150 mM NaCl, 0.1% SDS, 1% NP 40, pH 8;[38] or in 0.32 M sucrose solution with 50 mM Tris at pH 7.6[19] supplemented with protease inhibitors (ROCHE, Mannheim, Germany). The first were centrifuged at 14 000 *g* for 15 min, and the second at 1000 *g* for 10 min, at 4 °C. The supernatant was collected, corresponding to whole-tissue lysate and whole-tissue homogenate, respectively. For membrane isolation the whole-tissue homogenate was centrifuged at 14 000 *g* for 12 min, at 4 °C, the pellet is the membrane fraction. Protein was quantified using the BioRad Protein or DC Protein based on procedures previously described.[39,40]

Saturation-binding assays

The radioligand-binding experiments were performed as described[41] with membrane fractions. Briefly, [^{3}H]ZM 241385 binding (0–10 nM) was for 1 h with 20–35 μg of protein/well for striatum membranes and [^{3}H]DPCPX (0–10 nM) binding was for 2 h with 40–60 μg protein/well of hippocampal, 60–100 μg protein/well of cortex and 20–40 μg protein/well of striatum membranes. Specific binding was determined subtracting the non-specific binding, measured in the presence of 2 μM of XAC and normalized for protein concentration. Radioactivity was determined after 12 h with an efficiency of 55–60% for 2 min. All binding assays were performed in triplicate.

Immunoblotting

Lysates or homogenates were denatured with 5 × sample buffer (350 mM Tris pH 6.8, 30% glycerol, 10% SDS, 600 mM DTT and 0.012% Bromophenol blue, pH 6.8) and heated either at 95 °C for 5 min or at 60–70 °C for 30 min, respectively, and further processed as before.[29] A_{2A}R and $GABA_A$R antibodies (Upstate/Millipore, Temecula, CA, USA; 05–717 and 05–474) were at 1:2000, GR, MR (Santa Cruz Biotechnology, Heidelberg, Germany; sc-1004 and sc-11412) at 1:1000 and 1:200, NMDAR2B (Cell Signaling, Danvers, MA, USA; D15B3) at 1:1000 and glutamate receptor one (GluR1) (Millipore, 05–855) at 1:6000. Optical density was determined with Image-J software and normalized to the respective β-actin or α-tubulin band density.

Drugs

A_{2A}R-selective antagonist, 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo(4,3-e)(1,2,4)triazolo(1,5-c)pyrimidin-5-amine (SCH58261) and the non-selective adenosine receptor antagonist 8-(4-((2-minoethyl) amino)carbonylmethyloxyphenyl)xanthine (XAC) were purchased from Tocris Cookson, UK. These solutions were diluted in the assay solution from 5 mM stock aliquots made in DMSO stored at −20 °C. A_{2A}R-selective antagonist, (*E*)-8-(2-(3,4-dimethoxyphenyl)-vinyl)-1,3-diethyl-7-methyl-3,7-dihydropurine-2,6-dione (KW6002, istradefylline) was synthesized according to a published procedure.[42] The purity of the product was determined by HPLC analysis coupled to electrospray ionization mass spectrometry and was >98%. Adenosine deaminase (from calf intestine 10 mg/2 ml, EC 3.5.4.4) was from ROCHE; A_1R-selective antagonist, (propyl-^{3}H)8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX, specific activity 100 Ci per mmol) was from Amersham, Buckinghamshire UK, and A_{2A}R-selective antagonist, 4-(2-(7-Amino-2-(2-furyl)(1,2,4)-triazolo(2,3-a) (1,3,5)triazin-5-ylamino)ethyl)phenol, ([^{3}H]ZM 241385, specific activity 27.4 Ci per mmol) was from ARC, St Louis, MO, USA. All these drugs were diluted directly in the incubation solution each day. HRP-coupled secondary antibodies were from Santa Cruz Biotechnology. All other reagents used were of the highest purity available either from Merck or Sigma Aldrich, Madrid, Spain.

Statistics

Values presented are mean ± s.e.m. of *n* experiments. To test the significance of the differences between CTR and MS groups, an unpaired Student's *t* test was used. When comparing CTR, MS, CTR KW and MS KW groups a one-way ANOVA was used, followed by a Bonferroni's Multiple Comparison *post hoc* test. For the Sholl analysis of reconstructed neurons a repeated measures analysis was used. For the saturation binding curves an *F*-test was used to determine whether the competition curves were best fitted by one or two independent binding site equation and if the parameters obtained from the CTR and MS saturation curves (B_{max} and K_D) were different. For the analysis of the MWM acquisition curve and corticosterone circadian oscilation, the statistical differences were evaluated using two-way ANOVA repeated measures test. Values of $P<0.05$ were considered to be statistically significant.

Results

MS induces long-lasting regional effects in the brain

MS induces numerous changes in the brain particularly in the hippocampus.[43,44] The balance between mineralocorticoid and glucocorticoid receptors (MR and GR, respectively) can determine the impact of stress in different brain areas.[45] We quantified the levels of GR and MR in the hippocampus, cortex and striatum of adult animals previously subjected to MS (Figure 1a). MS has lead to a long-lasting decrease of the GR levels that was more evident in the hippocampus (0.74 ± 0.04 of CTR, $n=8$, $P<0.05$) than in cortex (0.86 ± 0.03 of CTR, $n=4$, $P<0.05$) or striatum (0.90 ± 0.03 of CTR, $n=4$; $P<0.05$). MR levels were not modified in any of the brain areas analyzed (Figure 1b). Concomitant changes on the levels of adenosine receptors were observed. Comparing with CTR, MS animals presented a 1.49 ± 0.04-fold increase ($n=9$; $P<0.05$) in the levels of A_{2A}R that was restricted to the hippocampus (Figure 1c). A hippocampal-specific decrease in the A_1R levels was also observed; B_{max} values were of 1202 ± 33 fmol per mg protein ($n=4$) for CTR animals and 1073 ± 23 fmol per mg protein for MS animals, ($n=4$; $P<0.05$; Figure 1d).

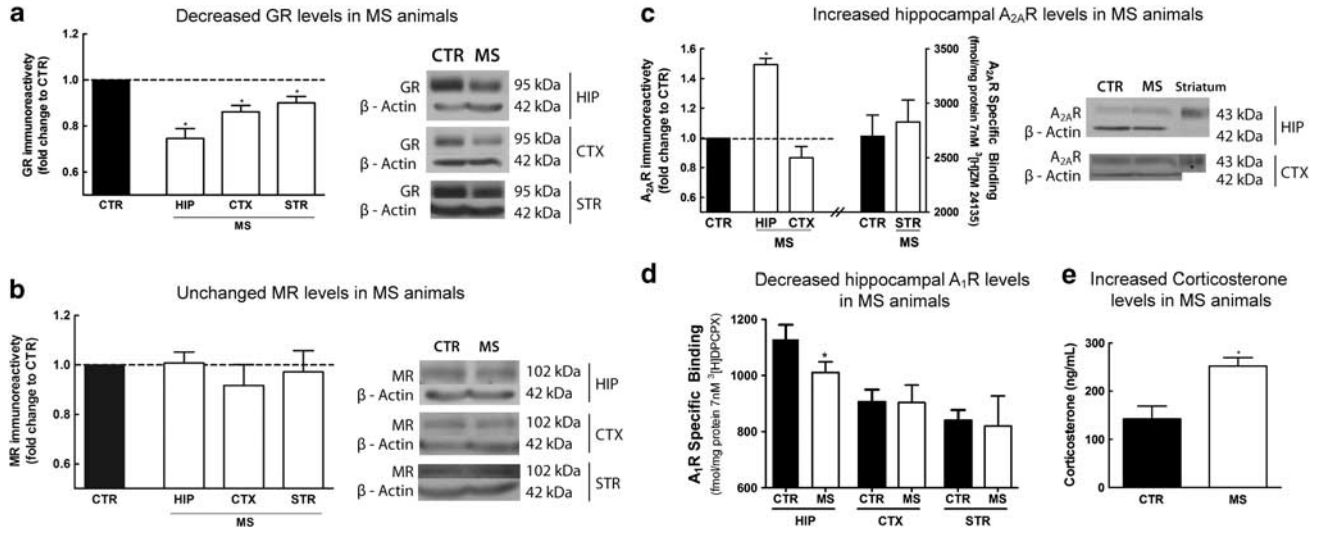

Figure 1 Region-specific effects of MS. MS induced region-specific changes in, GR (**a**) MR (**b**) $A_{2A}R$ (**c**) and A_1R (**d**) and an increase in plasmatic corticosterone levels (**e**). Protein levels of GR, MR (in all brain areas) and $A_{2A}R$ (in hippocampus and cortex) were evaluated by western blotting. Specific immunoreactivity was normalized to that of β-Actin or α-tubulin. For $A_{2A}R$ immunoreactivity 5 μg of striatum were used as positive control. Results are the mean ± s.e.m. of 3–9 experiments; $^*P<0.05$, comparing with CTR, calculated using an unpaired Student *t*-test. A_1R levels in all areas and $A_{2A}R$ levels in striatum were measured by saturation-binding curves with the A_1 or A_{2A} receptor selective antagonist [^{3}H]DPCPX or [^{3}H]ZM 24135, respectively. [^{3}H]DPCPX or [^{3}H]ZM 24135 (7 nM) were incubated with 20–100 μg of membranes in a final volume of 300 μl for 2 h/1 h at room temperature. The ordinates represent the specific binding obtained upon subtraction of the non-specific binding, determined in the presence of 2 μM of XAC, from total binding. Values are the mean ± s.e.m. of 4–5 experiments performed in triplicate. $^*P<0.05$ calculated using an *F*-test compared with CTR. Corticosterone levels in the morning period (8 am) were measured by radioimmunoassay using the rat corticosterone [^{3}H] kit. Results are mean ± s.e.m. of nine experiments; $^*P<0.05$ obtained using a unpaired Student *t*-test.

MS animals also presented a sustained increase in plasmatic corticosterone levels (Figure 1e).

Adenosine A_{2A} receptors are involved in synaptic changes induced by MS

To evaluate the impact of stress in synaptic transmission and plasticity, fEPSPs were measured in the CA1 area of the dorsal hippocampus. Basal synaptic transmission was accessed by performing I/O curves, whereas synaptic plasticity was evaluated by LTP induced by high frequency stimulation (100 Hz, 1 s).

The I/O curve was not modified by MS (Figure 2a, $n=3$). However, LTP magnitude (Figure 2b) was reduced in MS animals to 34.4 ± 2.7% from 50.7 ± 3.4% of potentiation obtained in CTR ($n=7$, $P<0.05$).

To evaluate whether the increase in A_{2A} adenosine receptors was involved in the impairments observed in synaptic plasticity, LTP was induced in the presence of SCH58261 (50 nM), a selective $A_{2A}R$ antagonist. The *ex vivo* blockade of $A_{2A}R$ reverted the LTP deficits induced by MS without affecting LTP magnitude in CTR animals (53.1 ± 3.7% and 57.3 ± 1.7% of potentiation, respectively, $n=4$–7, $P>0.05$; Figure 2c).

Moreover, the *in vivo* administration of the A_{2A}-selective antagonist KW6002 for 1 month to adult MS animals was also able to revert the LTP deficits observed (47.6 ± 3.9% of potentiation in MS KW animals, $n=4$, $P>0.05$ versus CTR; Figure 2d).

In order to better characterize the plastic changes observed in the electrophysiological studies, a 3D morphological analysis of dendritic arborizations of CA1 pyramidal neurons was performed. Data revealed a significant treatment effect in the total length of apical dendrites of pyramidal neurons ($F=7.371$, $P<0.001$), and in the total number of apical dendrite ramifications ($F=9.272$, $P<0.001$); *post-hoc* analysis showed that MS induced a significant decrease in the total length of apical dendrites when compared with CTR ($P<0.05$) (Figure 2e). Similarly, MS pyramidal neurons had significantly less ramifications in apical dendrites when compared with CTR ($P<0.05$). Both parameters were restored by KW6002 treatment ($P>0.05$ versus CTR). There were no significant differences in the structure or number of basal dendrites. Sholl analysis showed coherent changes in the pattern of ramification ($F=5.691$, $P<0.001$). The changes in apical dendrite arborizations observed in MS animals when compared with CTR ($P<0.05$) were reverted by KW6002 (Supplementary Figure S2).

To further evaluate the consequences to the overall morphology we undertook an estimation of hippocampal formation volumes. Our data revealed that neither MS nor KW6002 treatment significantly affected volumetric estimates (Supplementary Table S1).

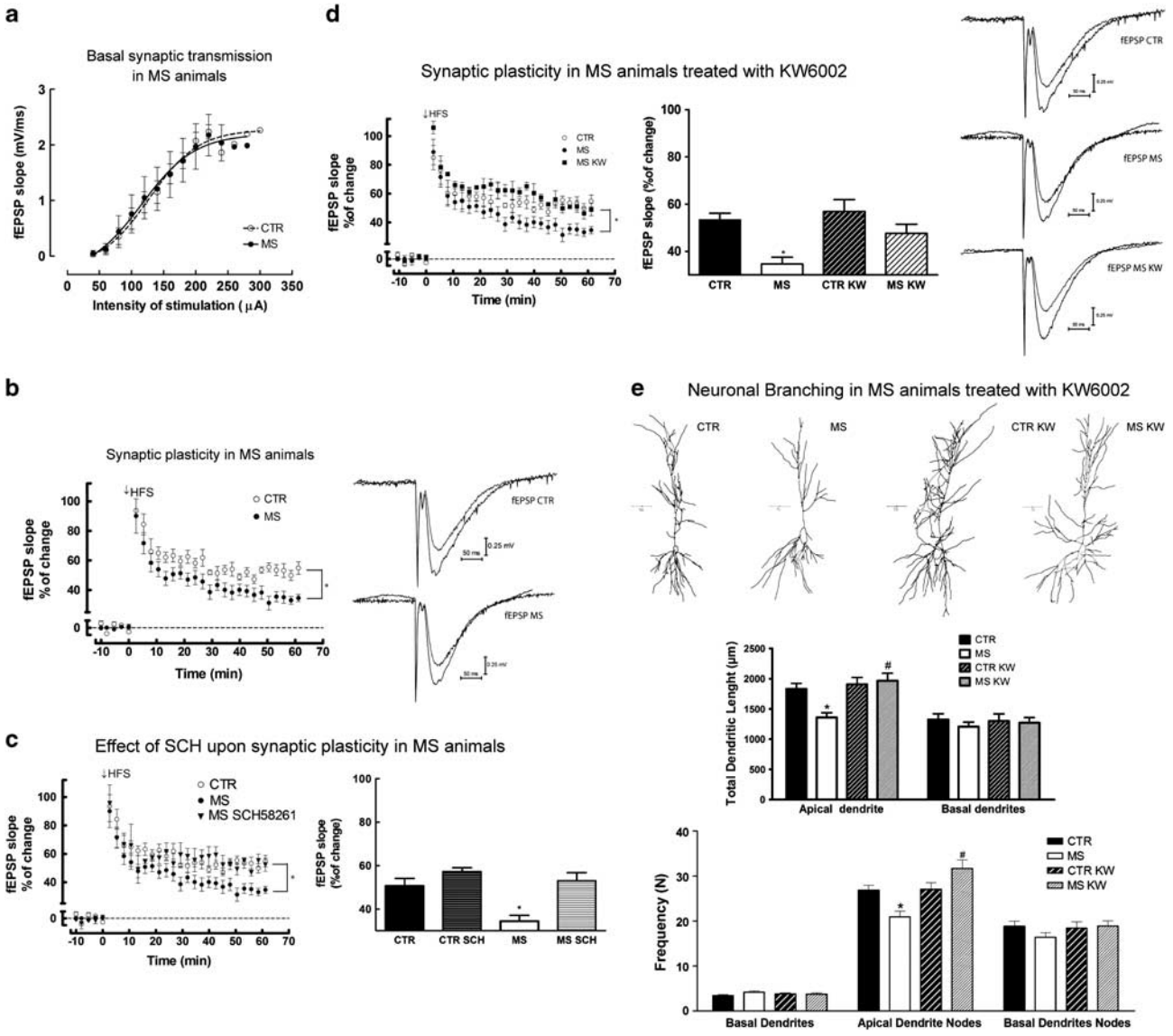

Figure 2 Involvement of adenosine A_{2A} receptors in the synaptic changes induced by MS. (**a**) I/O curves performed to evaluate synaptic transmission in CTR and MS animals and (**b**) LTP (high frequency stimulation, 100 Hz,1 s), used to evaluate synaptic plasticity. Representative recordings of the fEPSPs obtained both for CTR and MS animals before LTP induction and in the last 10 min are presented. The effect of SCH58261 (50 nM) application for 30 min before LTP induction and throughout the protocol is shown in (**c**). The outcome of KW6002 treatment upon LTP is in (**d**) with representative recordings of the fEPSPs obtained for CTR, MS and MS + KW6002 animals, before LTP induction and 1 h after LTP. Bar graphs are obtained by making the average of the last five timepoints of each experiment. Results are the mean ± s.e.m. of 3 (**a**) or 4–7 experiments $^{*}P<0.05$, comparing with CTR. (**e**) Administration of KW6002 reverses dendritic atrophy induced by MS in CA1 pyramidal neurons. Upper panel depicts representative schematics of 3D reconstructions of CTR, MS, CTR KW and MS KW CA1 neurons. $^{*}P<0.05$, comparing with CTR, $^{\#}P<0.05$, comparing with MS, calculated using a one-way ANOVA followed by a Bonferroni's Multiple Comparison Test.

Oral administration of a selective A_{2A} receptor antagonist reverts the stress-induced anxious behavior and learning-deficits

We then evaluate the extent to which $A_{2A}R$ are involved in the stress-induced behavior alterations, by the administration of the $A_{2A}R$-selective antagonist, KW6002, to adult MS animals.

Anxious behavior and hippocampal-dependent memory were evaluated by the EPM and the MWM paradigms, respectively. In the EPM, MS animals presented a higher anxious-related behavior (spent less time in the open arms, 11.4 ± 2.0% versus 28.9 ± 5.3% in CTR, $n=8$–11, $P<0.05$), validating the MS stress induction. The hyperanxious behavior in MS animals was reverted upon treatment with KW6002 (time in open arms: 27.3 ± 4.4%, $n=8$, $P>0.05$ versus CTR; Figure 3a). KW6002 treatment by itself had no effect in the anxious behavior of CTR

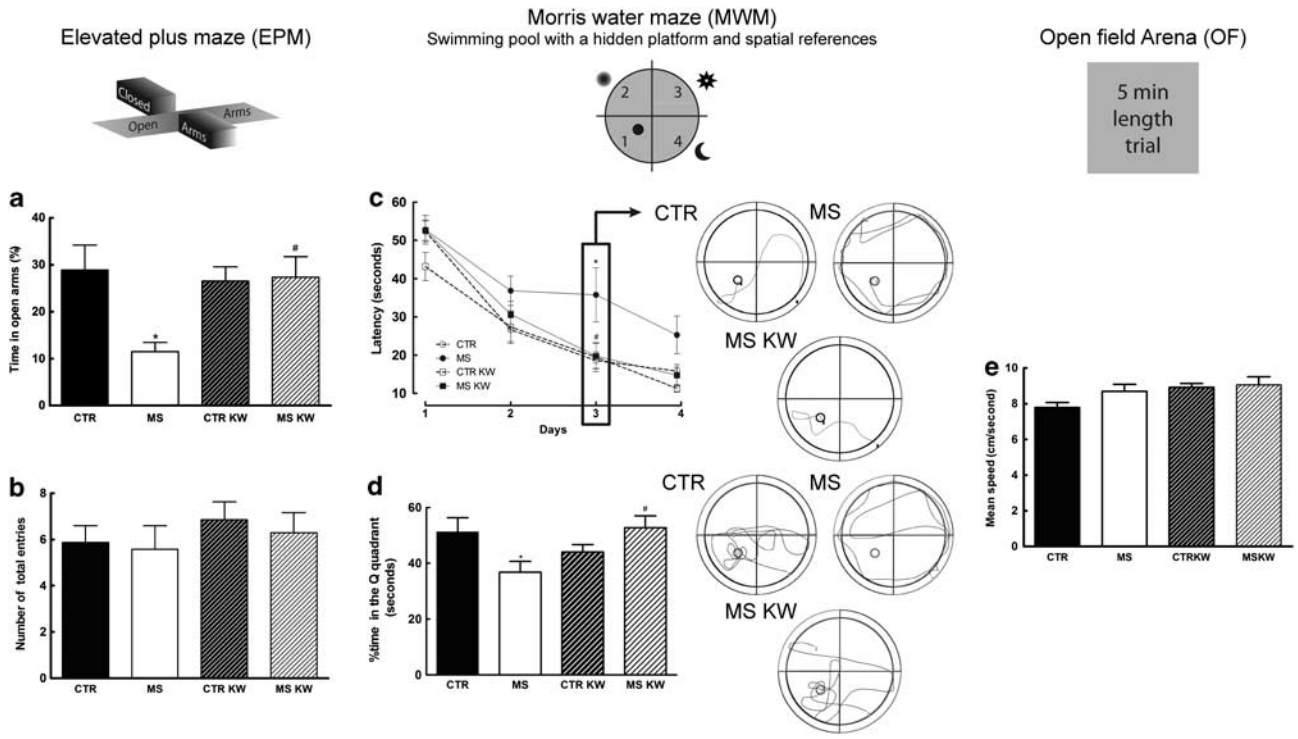

Figure 3 Administration of KW6002 reverts the stress-induced anxious behavior and learning deficits. Anxious behavior (**a**, **b**) and locomotor activity (**e**) were evaluated by the elevated-plus-maze-test and open-field, respectively. Hippocampal-dependent memory performance was assessed by the MWM test, in which acquisition (**c**) and retention (**d**) were evaluated. Results are the mean ± s.e.m. of 6–9 animals; $^{*}P<0.05$, comparing with CTR, $^{\#}P<0.05$ comparing with MS, calculated using two-way ANOVA repeated measures (**a**) or one-way ANOVA followed by a Bonferroni's Multiple Comparison Test.

animals ($26.5 \pm 3\%$, $n=7$, $P>0.05$), neither had an impact in locomotor performance in EPM (Figure 3b). On the MWM, the learning ability (Figure 3c) of MS animals was impaired, so that at day three MS performed worse than CTR animals ($F(3,132)=8.56$, $n=6$, $P<0.0001$). These deficits were reverted by blocking $A_{2A}R$ *in vivo* ($n=10$, $P<0.05$). The retention ability of MS animals was also compromised, as in the probe test MS animals spent less time in the platform quadrant than CTR ($36.8 \pm 3.9\%$, $n=8$; $P<0.05$ versus $51.3 \pm 5.0\%$, $n=7$; Figure 3d). When treated with the $A_{2A}R$ antagonist the retention ability of MS animals was restored ($52.8 \pm 4.2\%$ of the time in the platform quadrant; $n=8$, $P>0.05$; Figure 3d). KW6002 by itself had no effect in the performance of CTR animals.

$A_{2A}R$ are highly abundant in striatum exerting important effects in motor control.[46] To evaluate directly locomotor activity, the open-field arena test was used. In accordance with data obtained in the EPM, neither MS nor the KW6002 treatment induced changes in the locomotor performance of the animals, as no alterations were observed in the mean speed on the open-field arena (Figure 3e).

Oral administration of a selective A_{2A} receptor antagonist re-establishes stress-induced modifications on synaptic markers

Given the positive effects of the *in vivo* KW6002 treatment in behavior, in *ex vivo* synaptic plasticity and in neuronal morphology, we next explored the molecular changes that could underlie the observed therapeutic effects. The levels of $A_{2A}R$ and GR were measured in the hippocampus of CTR and MS animals treated with KW6002. Given the role of AMPA, $GABA_A$ and NMDA receptors in synaptic transmission and plasticity, AMPA-GluR1, $GABA_A$-$\beta_{2/3}$ and NMDAR2B subunits levels were also evaluated. AMPA-GluR1 levels in MS animals were significantly decreased comparing with CTR (0.81 ± 0.02, $n=9$, $P<0.05$; Figure 4a). These values were re-established by KW6002 (0.98 ± 0.03, $n=4$, $P>0.05$ versus CTR). $GABA_A$-$\beta_{2/3}$ levels decreased in MS animals (0.78 ± 0.02, $n=8$; $P<0.05$; Figure 4b) and increased to 1.15 ± 0.06 of CTR ($n=6$, $P<0.05$) upon KW6002 treatment. The levels of the NMDAR2B subunit were not altered by MS nor by KW6002 treatment ($n=5$; Figure 4c). Furthermore, the increased levels of $A_{2A}R$ observed in MS animals (1.49 ± 0.04-fold to CTR, $n=9$; $P<0.05$) were maintained in MS KW animals (1.56 ± 0.05-fold to CTR, $n=5$, $P>0.05$; Figure 4d). The KW6002 administration alone increased the levels of $A_{2A}R$ to 1.25 ± 0.09 of CTR ($n=5$, $P<0.01$; Figure 4d), as could be expected from a chronic administration of receptor antagonist. The GR levels (Figure 3e) were not changed by KW6002 in CTR animals ($n=7$, $P>0.05$); however, when KW6002 was administered to MS animals the levels of GR increased to values similar to CTR (1.033 ± 0.04, $n=6$, $P>0.05$).

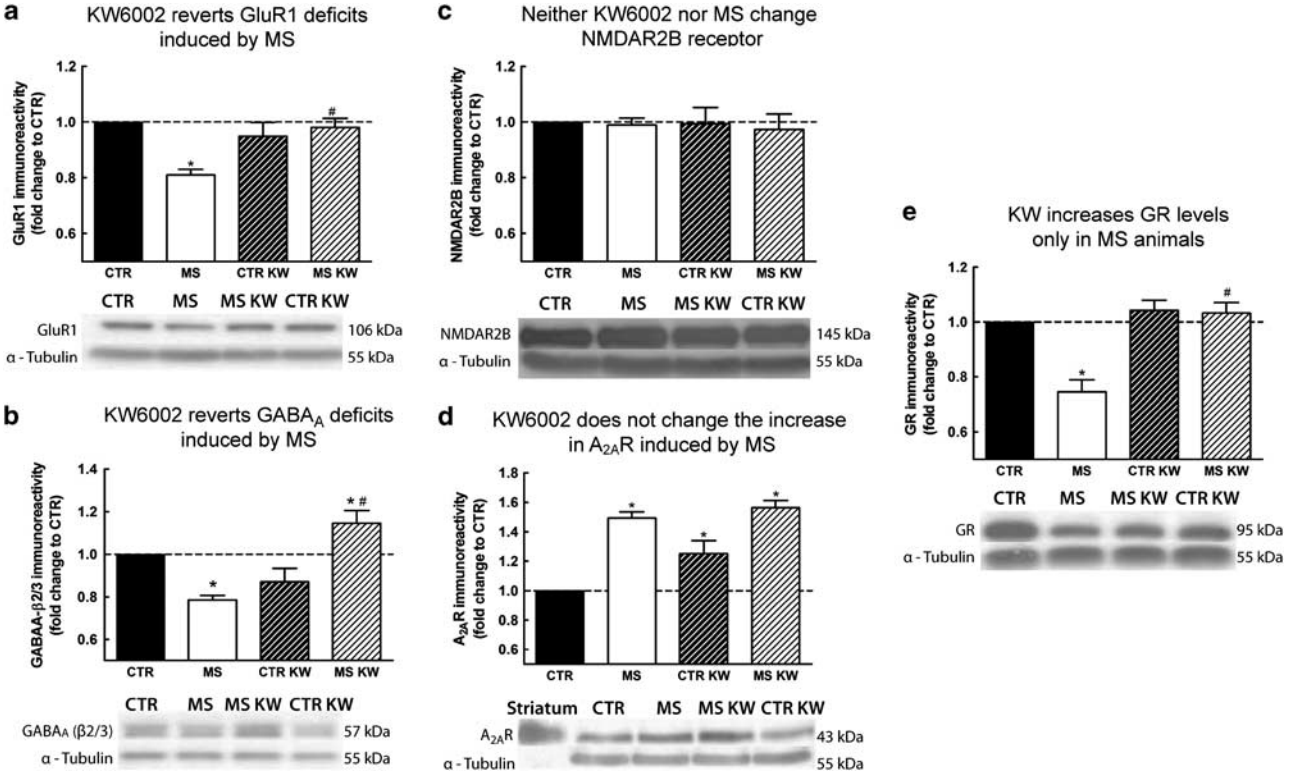

Figure 4 Administration of KW6002 reverts the synaptic changes induced by MS. The effects of the treatment with KW6002 in the levels of GluR1 subunit of AMPA receptors (**a**), β2/3 subunit of $GABA_A$ receptors (**b**), NMDAR2B (**c**), A_{2A}R (**d**), and GR (**e**) were evaluated by western blotting. Specific immunoreactivity normalized to that of α-tubulin. Results are the mean ± s.e.m. of 4–9 experiments; $^*P<0.05$, comparing with CTR and $^{\#}P<0.05$, comparing with MS, calculated using a one-way ANOVA followed by a Bonferroni's Multiple Comparison Test.

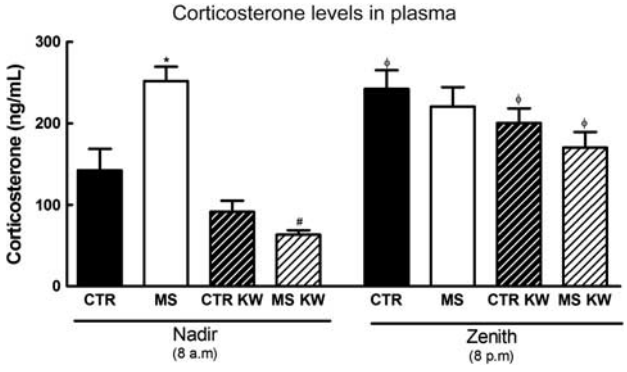

Figure 5 The KW6002 administration re-establishes the corticosterone circadian oscillation. Corticosterone levels in the plasma measured at nadir and zenith. Results are mean of 6–9 animals.$^*P<0.05$, comparing with CTR, $^{\#}P<0.05$ comparing with MS, $^{\phi}P<0.05$, comparing with am values, calculated using a one-way ANOVA followed by a Bonferroni's Multiple Comparison Test.

The A_{2A}R antagonist re-establishes the corticosterone circadian oscillation

As the blockade of A_{2A}R reestablishes the GR levels in the hippocampus, we hypothesized that this could involve a regulation of the HPA-axis function, which is compromised due to the early life stress.[14] HPA-axis activity was evaluated by measuring circadian changes in plasmatic corticosterone levels (Figure 5).

CTR animals had the expected circadian oscillation, with corticosterone levels significantly elevated at 8 pm comparing with those measured at 8 am MS animals present significantly higher corticosterone levels already at 8 am ($234 \pm 13\,ng\,ml^{-1}$ versus $142 \pm 27\,ng\,ml^{-1}$; $P<0.05$, $n=9$) comparing with CTR at the same time of the day, and the absence of a circadian oscillation. Animals treated with KW6002 had a restored circadian variation, with plasmatic corticosterone levels at 8 am similar to CTR ($63 \pm 5\,ng\,ml^{-1}$), ($F(3,24)=9.04$, $P=0.0003$). KW6002 alone did not affect corticosterone levels, neither at zenith nor at nadir.

Discussion

The data now reported reveal that adenosine A_{2A} receptor activation is directly involved in the stress deleterious effects in the brain. We show, for the first time, that the administration of a selective adenosine A_{2A} receptor antagonist reverts the long-lasting consequences of stress on spatial memory, synaptic plasticity and neuronal morphology in the hippocampus. Moreover, our data indicate that these effects are

associated with the re-establishment of the HPA-axis activity.

An imbalance in adenosine receptors has been observed in multiple conditions,[21] particularly with progressive aging,[18,19] which has consequences to their modulatory effects.[19,41] In the aged rat brain, adenosine A_1 receptor density is decreased,[47] particularly in hippocampus and cortex.[18] However, A_{2A} receptor levels are differently affected: they decrease in striatum, but in contrast there is an increase in their expression in cortical and hippocampal areas.[18] As we now show, the changes in adenosine receptor levels induced by MS, follow a close pattern to the one occurring in the aged brain, that is, an increase in A_{2A} and a decrease in A_1 receptor levels. The modifications observed are, however, restricted to the hippocampus, probably due to the changes in GR levels that are more profound in this brain area. Thus, as observed in aging,[19,48] MS induces a decrease in GR levels, increasing the MR/GR ratio, an increase in plasma corticosterone levels and changes in adenosine receptor levels. Thereupon our data reinforce the hypothesis that stress is associated with an early aging in the hippocampal area.[2,49] Different brain regions have a distinct vulnerability to stress due to the differential expression of GR and MR in the brain.[50] In the hippocampus, corticosterone is able to trigger signaling pathways activated by both GR and MR due to their particular high-affinity ratio for GR, which does not occur in other brain areas.[50] This confers to the hippocampus a particular susceptibility to stress effects and consequent deficits. Additional region-specific effects were reported previously, such as alterations in $GABA_A$ receptor levels and MAP kinase activity.[51,52]

We have observed a sustained increase in the plasmatic levels of corticosterone, a feature that is also shared with ageing.[53] Such an increase is usually associated to a downregulation in GR,[11,54,55] as a way to limit their action. This is generally an isolated and reversible effect, reverted whenever the plasma levels of corticosterone return to baseline.[55] However, as we show, MS animals exhibit elevated plasma corticosterone levels throughout life and an associated sustained downregulation of GR in the hippocampus. These receptors regulate memory and synaptic plasticity.[45,56] Accordingly, we found that LTP is impaired in MS animals and this is accompanied by a poorer performance in a spatial memory task, the MWM. The observed changes in synaptic plasticity can be related to the altered levels in $GABA_A$ and AMPA receptors, reported here. Others have described that MS induces a decrease in markers of synaptic plasticity, such as NCAM or synaptophysin,[43] as well as in the levels of NMDAR2B, AMPAGluR1 and GluR2[44] in the hippocampus. Changes now observed in glutamate receptor levels had, however, no impact upon basal synaptic transmission, possibly because they are accompanied by a decrease in $GABA_A$ receptors, which will result in a final compensatory balance in order to maintain homeostasis.

The observed impairments in LTP were overcome by blocking adenosine A_{2A} receptors. These receptors are known to have stimulatory effects on basal synaptic transmission in the hippocampus[17,41] by promoting glutamate release,[57] and were recently shown to potentiate LTP when exogenously activated.[58] The protective effect of administering A_{2A} receptor antagonists *in vivo* has already been described.[21] By contrast, we have observed before that, in particular conditions, the acute treatment of slices with SCH58261 may instead cause a LTP drop. This is particular to overexcitability conditions, such as ageing, in which LTP is enhanced[29,59] due to an age-induced shift in A_{2A} receptor signaling.[19,41] However, in more chronic patho-physiological situations, in which LTP is decreased, the SCH58261 is able to promote its restoration,[60] in accordance with what we now report for stress-induced deficits.

More importantly, the chronic administration of a selective antagonist, KW6002, for 1 month, clearly re-established the MS-driven impairment in LTP. However, the A_{2A} receptor antagonist did not alter LTP in CTR animals, whereas clearly promoting the recovery of the impaired LTP, but only in MS animals. This suggests that, rather than having a direct effect on glutamatergic transmission, A_{2A} receptors may be instead modulating the GR-mediated effects. Indeed, we have recent data showing the ability of A_{2A} receptors to influence GR transcriptional activity and nuclear translocation.[61] Thus, the chronic blockade of A_{2A} receptor may decrease GR transcriptional activity and thereby the overall GR-driven effects.

Genetic deletion of A_{2A} receptors affects anxiety and aggressive behavior,[62] and this constituted the first evidence that A_{2A} receptors could be implicated in stress. The subsequent report that A_{2A} receptor antagonists applied before and during a single episode of acute stress prevented synaptic loss,[22] suggested that A_{2A} receptors overactivation could underlie the genesis of stress-induced changes. Nonetheless, the question whether this overactivation is a consequence of the stress paradigm or a triggering factor to the observed deficits has never been addressed before.

We now explored the possibility that blocking the action of A_{2A} receptors would restore pre-existing stress-associated impairments. The advantage of using KW6002 over other antagonists for A_{2A} receptors is its enhanced bioavailability, permeability to the brain blood barrier, having a longer half-life and high affinity and selectivity towards A_{2A} receptors.[27] Additionally, KW6002 has undergone clinical trials for Parkinson's and therefore its safety has been established.[63] We now report that oral administration of KW6002, for 1 month, to adult animals previously subjected to MS, reestablishes impaired hippocampal-dependent memory, synaptic plasticity and morphology, and reverts the anxious behavior. The learning ability of MS animals was restored by the treatment, as well as the retrieval, evaluated by the time spent in the previous retained platform

quadrant. This is associated with a re-establishment of the hippocampal CA1-induced LTP. The insertion of AMPA receptors containing $GluR_1$ subunit is a constitutive part of LTP induction[64] and is modulated by GR.[65] We found that GluR1-subunit is decreased by MS, which may explain the decreased LTP. Moreover, the LTP re-establishment is accompanied by a concomitant restoration of GluR1 levels upon KW6002 treatment. Accordingly, MS leads to a decrease in apical dendritic length, as described using other stress models,[66] but this structural effect is reverted by the blockade of A_{2A} receptors.

The HPA-axis maintains the physiological circadian oscillation of corticosterone levels, which reach their maximum at zenith (8 pm) and the minimum at nadir (8 am), for rodents.[67] The hippocampus is crucial in the negative feedback required to limit HPA-axis activation, particularly in stressful situations.[1,2] However, this function can be compromised when glucocorticoid levels are persistently high as in chronic stress, aging or in psychopathologies.[48] The observation that the A_{2A} receptor antagonist was able to re-establish the decreased GR levels in the hippocampus lead us to test whether the observed effects were related to a modification of the HPA axis, by measuring the circadian levels of corticosterone in plasma. MS animals present not only higher plasmatic levels of corticosterone, but also an impaired circadian fluctuation. Corticosterone levels were chronically higher in MS animals and did not decrease along the night. This is probably associated to an impaired inhibition of the HPA-axis, which is consequence of a decrease in hippocampal GR levels. Interestingly, by blocking A_{2A} receptors, the basal levels of corticosterone were re-established, so as the circadian rhythm and the GR levels in the hippocampus. Altogether, these data suggest that A_{2A} receptors have a role in the regulation of the HPA-axis, either directly or by regulating hippocampal function. This effect may be due to interference with the release of corticotrophin-releasing hormone, adrenocorticotrophin, which is known to be affected by adenosine,[68] or by modulating glucocorticoids. The beneficial effect resulting from A_{2A} receptor antagonism may derive instead from a re-establishment of hippocampal excitability, which in turn would restore the inhibitory tonus onto the HPA-axis. Overall, the blockade of adenosine A_{2A} receptors by KW6002 has a beneficial effect in overcoming the hippocampal-related deficits induced by MS. Interestingly, this effect of KW6002 *in vivo* does not result from a decrease in $A_{2A}R$ levels, which remain high in MS animals under KW6002. Indeed, it would be unlikely that KW6002 would cause a decrease in $A_{2A}R$ levels as prolonged blockade of receptors usually leads to either no change or upregulation of receptor levels due to compensatory mechanisms following restraining from receptor activation by the endogenous ligand. Remarkably, the findings that blockade of $A_{2A}R$ overcomes the synaptic and memory deficits associated to MS, strongly suggests that A_{2A} receptors overactivation is the cause rather than the consequence of the herein reported changes associated with chronic stress.

In conclusion, our results show, for the first time, that the changes induced by stress are reverted by the *in vivo* blockade of A_{2A} receptors. Moreover, they imply a role of $A_{2A}R$ in the HPA-axis regulation revealing that its blockade is efficient in re-establishing the compromised HPA-axis, which has clinical implications for the treatment of psychopathologies. This provides a potential alternative to the established therapies against stress-related pathologies, by targeting a modulatory system rather than interfering directly with neurotransmitters, and thereby limiting the associated side-effects.

Conflict of interest

The authors declare no conflict of interest.

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