Optogenetic activation of intracellular adenosine $A_{2A}$ receptor signaling in the hippocampus is sufficient to trigger CREB phosphorylation and impair memory

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INTRODUCTION

Recently, six longitudinal prospective studies have established an inverse relationship between caffeine consumption and the risk of developing cognitive impairments in aging and Alzheimer’s disease through the antagonism of adenosine $A_{2A}$ receptors ($A_{2A}$Rs). To test if $A_{2A}$R activation in the hippocampus is actually sufficient to impair memory function and to begin elucidating the intracellular pathways operated by $A_{2A}$R, we have developed a chimeric rhodopsin-$A_{2A}$R protein (opto$A_{2A}$R), which retains the extracellular and transmembrane domains of rhodopsin (conferring light responsiveness and eliminating adenosine-binding pockets) fused to the intracellular loop of $A_{2A}$R to confer specific $A_{2A}$R signaling. The specificity of the opto$A_{2A}$R signaling was confirmed by light-induced selective enhancement of cAMP and phospho-mitogen-activated protein kinase (p-MAPK) (but not cGMP) levels in human embryonic kidney 293 (HEK293) cells, which was abolished by a point mutation at the C terminal of $A_{2A}$R. Supporting its physiological relevance, opto$A_{2A}$R activation and the $A_{2A}$R agonist CGS21680 produced similar activation of cAMP and p-MAPK signaling in HEK293 cells, of p-MAPK in the nucleus accumbens and of c-Fos/phosphorylated-CREB (p-CREB) in the hippocampus, and similarly enhanced long-term potentiation in the hippocampus. Remarkably, opto$A_{2A}$R activation triggered a preferential p-CREB signaling in the hippocampus and impaired spatial memory performance, while opto$A_{2A}$R activation in the nucleus accumbens triggered MAPK signaling and modulated locomotor activity. This shows that the recruitment of intracellular $A_{2A}$R signaling in the hippocampus is sufficient to trigger memory dysfunction. Furthermore, the demonstration that the biased $A_{2A}$R signaling and functions depend on intracellular $A_{2A}$R loops prompts the possibility of targeting the intracellular $A_{2A}$R-interacting partners to selectively control different neuropsychiatric behaviors.

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intriguing possibility that the interaction of A2AR intracellular domains with different GIPs may dictate the biased A2AR signaling in different cells. However, the inability to control intracellular GPCR signaling in vivo in a precise spatiotemporal manner has prevented translation of in vitro profiles of A2AR signaling into behavior in intact animals. To determine if the abnormal activation of A2AR signaling in the hippocampus is sufficient to impair memory function in freely behaving animals and to begin elucidating the nature of the biased A2AR signaling in different brain regions, we have developed a chimeric rhodopsin-A2AR protein (optoA2AR): this merges the extracellular and transmembrane domains of rhodopsin conferring light responsiveness and the intracellular domains of A2AR conferring specific A2AR signaling, to investigate the biased A2AR signaling in defined cell populations of freely behaving animals in a temporally precise and reversible manner. Furthermore, the selective retention of only the intracellular domains of A2AR in optoA2AR chimaera permits a critical evaluation of its particular role controlling the biased A2AR signaling. After validating the specificity and physiological relevance of light-induced optoA2AR recruitment of A2AR signaling in human embryonic kidney 293 (HEK293) cells, mouse brain slices and in freely behaving animals, we exploited its unique temporal and spatial resolution to provide direct evidence that the activation of intracellular A2AR signaling selectively in the hippocampus is sufficient to recruit cAMP and phosphorylated-CREB (p-CREB), and alter synaptic plasticity and memory performance. Our findings also provide a direct demonstration that the intracellular control of the biased A2AR signaling in striatal and hippocampal neurons triggers distinct signaling and behavioral responses.

MATERIALS AND METHODS

Design and construction of the optoA2AR vector

We have developed a chimeric protein optoA2AR by replacing the intracellular domain of rhodopsin with those of the A2AR (Figure 1a). We first aligned the conserved residues of the sequence of the A2AR (NCBI accession no. NM_000675.4) with the bovine rhodopsin (NCBI accession no. P02699), and identified the intracellular loops 1, 2 and 3 of A2AR to be exchanged with the intracellular loops of rhodopsin (Figure 1a). Then, we constructed a fusion gene encoding a chimera (optoA2AR) by replacing the intracellular loops 1, 2 and 3 of the C-terminal of rhodopsin with those of A2AR and by adding the N-terminal of bovine rhodopsin (TETSQVAPA) to the N terminus of mCherry (with its start codon deleted) with a linker (5′-GGCCGCCGCCC-3′) for fluorescence detection of optoA2AR in cells and tissues. The optoA2AR construct was cloned into a pcDNA3.1 vector at the EcoRI–Xhol sites.

Transfection and detection of optoA2AR in HEK293 cells

HEK293FT cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the Invitrogen protocols, and the red fluorescence of mCherry was detected 48 h after transfection. To study optoA2AR signaling, all-trans-retinal (25 μM) was added and incubated at 37 °C with 5% atmospheric CO₂ for 60 min. The cells were then illuminated with 500 nm light at 3 mW mm⁻² using a high-intensity fiber-coupled light source (OSL1-EC; Thorlabs, Newton, NJ, USA). The cells were then fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS). Cells were blocked with 10% normal goat serum and incubated with anti-A2AR (Santa Cruz, Dallas, TX, USA; 1:500) or anti-P-MAPK antibodies (Cell Signalling, Danvers, MA, USA; 1:200) overnight at 4 °C. After extensive washes with phosphate buffer saline, cells were incubated with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) for 60 min at room temperature.

Assessment of optoA2AR signaling in HEK cells

For bioluminescent assay, HEK293 cells, kept in 96-well plates, were illuminated (500 nm, 3 mW mm⁻²) for 60 sec. The cells were then lysed 30 min after cessation of light illumination to analyze CAMP using CAMP-Glo™ assay (Promega, Madison, WI, USA). cGMP by HTRF cGMP assay and IP1 by HTRF IP1 assay kit (CisBio, Sunnyvale, CA, USA.). For western blot, cells were isolated at 10 min after light illumination, using a PARIS Kit (Invitrogen). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gels) and transferred onto an Immobilon-P PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked for 1 h in Tris-buffered saline with Tween-20 (TBS-T, pH 7.6) containing 5% non-fat dry milk powder and thereafter incubated overnight at 4 °C with antibodies against mitogen-activated protein kinase (MAPK, Danvers, MA, USA) (Cell Signalling, 1:500), phospho-MAPK (p-MAPK) (Cell Signalling; 1:500) or A2AR (Santa Cruz; 1:1000). The membranes were then probed with different conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA; 1:3000) at room temperature for 1 h, followed by washing in TBS-T.

Virual production

Recombinant AAV vectors were constructed with a transgene cassette encoding CaMKIIα promoter by cloning the optoA2AR-mCherry into pAAV-CaMKII-eNPH 3.0-EYFP (Addgene, Cambridge, MA, USA) using BamHI– HindIII assisted by GeneScript (Piscataway, NJ, USA). Viral particles were packaged and purified by vector core at the University of North Carolina (Chapel Hill, NC, USA) for serotype 5, and titers were 1.5 × 10¹² particles per ml. The AAVs-CaMKII-mCherry was purchased from the University of North Carolina at Chapel Hill as ‘control’ virus with a titer of 2.0 × 10¹² particles per ml.

Animals

All procedures were in accordance with the National Institutes of Health Guide and with EU guidelines for the care and use of laboratory animals and approved by the IACUC at the Boston University School of Medicine (Boston, MA, USA; AN no.14684) and by University of Coimbra (Coimbra, Portugal; ORBEA-78-2013 ethical approval). C57BL/6 mice at 11–13 weeks old (weighing 24–28 g) were used in the study. The A2AR knockout mice on a C57BL/6 background were described previously.

Animal surgery

CNS injection and cannulae implantation

Animals were anesthetized with Avertin (10 ml kg⁻１ intraperitoneally). Under a stereotactic frame, a midline scalp incision was made and a ~1 mm diameter craniotomy was drilled to the right nucleus accumbens (NAc; AP, +1.1 mm; ML, ± 1.4 mm; DV, +4.5 mm) or the hippocampus (AP, −2.2 mm; ML, ± 1.5 mm; DV, +2.3 mm). To express optoA2AR in neurons of the NAc or hippocampus, we injected 1.0 μl of AAVS-CaMKII-optoA2AR-mCherry virus at 0.75 μl μl⁻1 of AAVS-CaMKII-mCherry (control) virus at 0.1 μl μl⁻1. To activate the endogenous A2AR in the NAc and hippocampus, we injected 2.0 μl of the A2AR agonist CGS21680 (0.5 μg μl⁻1) at 0.1 μl min⁻¹ using an automated syringe pump using a beveled 33-gauge needle. Following the injection, the needle was left for 5 min to allow the drug diffusion. The mice were killed after 15 min and 1 h after injection and processed for immunohistochemistry. We also bilaterally implanted guide cannulae (Plastics One, Roanoke, VA, USA) on the skull through the craniotomy, to bilaterally target the two hippocampi or the two NAc, which were secured using dental cement (Lang Dental, Wheeling, IL, USA). Animals were allowed to recover for at least 2 weeks before the experiment.

Electrophysiological recordings of synaptic plasticity in hippocampal slices

The experiments were carried out as described previously. The hippocampus was dissected in an ice-cold Krebs solution and slices (400 μm) were prepared with a McIlwain chopper. Individual slices were transferred to a submersion recording chamber (1 ml capacity) and continuously superfused at a rate of 3 ml min⁻¹ with gassed Krebs solution (composition in mM: NaCl 124, KCl 3, NaH₂PO₄ 1.25, glucose 10, NaHCO₃ 26, MgSO₄ 1, CaCl₂) kept at 30.5 °C. A bipolar concentric electrode was placed onto the Schaffer collateral/commissural pathway fibers and stimulated every 20 s with rectangular pulses of 0.1 ms with a Grass S44 pulse generator (PSIU6; Grass, West Warwick, RI, USA). The orthodromically evoked field excitatory postsynaptic potentials were recorded through an extracellular glass microelectrode (filled with 4 M NaCl; resistance: 2–4 MΩ) placed in the stratum radiatum of the CA1 area. The high-frequency stimulation (HFS) protocol used to induce long-term potentiation (LTP) consisted of a 100 Hz train during 1 s. Light stimuli consisted of...
3000 light pulses (465 nm, 50 ms pulse width, ~3–5 mW mm\(^{-2}\) power density) during a total period of 300 s, applied through a Plexibright LD-1 LED module with a 465 nm Blue (Plexon, Dallas, TX, USA) and the optic fiber was placed over the slice between the stimulation and recording electrodes. Light stimuli were triggered 300 s before the delivery of the HFS protocol. The A2AR agonist CGS21680 (30 nM; Tocris, Ballwin, MO, USA) was added to the superfusion solution at least 20 min before the HFS protocol onwards. LTP amplitude was quantified as the percentage change between two values: the average slope of the five potentials taken between 50 and 60 min after the induction protocol in relation to the average slope of the field excitatory postsynaptic potentials measured during 15 min that preceded the induction protocol.
Optogenetic activation of optoA2AR signaling in the brain

A 200 μm core fiber (Thorlabs, Newton, NJ, USA) was used for optical stimulation via a patch cable connected to a 473 nm DPSS laser (100 mW; Shanghai Laser & Optics Century, Shanghai, China). The power density at the fiber tip was ~3–5 mW mm−2 and light was delivered with 50 ms pulse width. For assessment of optoA2AR signaling, mice were killed following 10 min optical stimulation. For behavior assessments, optical stimulation was delivered specifically according to different behavioral test (see below).

Behavioral tests

For the open-field test, mice were placed in the center of a white, dimly light open-field chamber (40 × 40 cm²) and allowed to freely explore the environment. The center of the open-field was defined as >10 cm apart from all four walls. Off-On-Off episodes of light stimulation were used and each block lasted 5 min, for a total of 15 min. Locomotor activity was detected with a video camera and analyzed with AnyMaze Video Tracking System (Stoelting, Wood Dale, IL, USA). The Y-maze test for recognition memory task was based on exploration of novelty as described previously. The test consisted of two trials, separated by a 1-h time interval. During the first trial (acquisition phase), one arm of the Y-maze was closed and mice were allowed to explore the remaining two arms for 10 min with light ‘OFF’, with several visual clues on the walls of the room. During the second trial (retrieval phase), the mice had access to the three arms for a 5-min period with light ‘ON’. During this period, the time spent in each arm and the total locomotor activity of each mouse was measured by a video-tracking system (Smart; Bioseb Chaville, Chaville, France).

Immunohistochemistry

Following agonist injection or light stimulation, mice were transcardially perfused with ice-cold 4% paraformaldehyde in PBS (pH 7.4) after termination of the light stimulation. Brains were postfixed and coronal sections with 30–40 μm were cut and processed for immunohistochemistry. Free-floating sections were washed in PBS and then incubated for 30 min in 0.3% Triton X-100 and 3% normal donkey or goat serum. Primary antibody incubations were conducted overnight in 0.01% Triton X-100 and 3% normal donkey serum or A2AR (Santa Cruz; 1:200), p-MAPK (Cell Signaling; 1:200), p-CREB (Cell Signaling; 1:200) or c-fos (Santa Cruz; 1:300). Sections were then washed with PBS and incubated for 1 h at room temperature with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes; 1:200). Slides were then washed and mounted on slides with Vectashield mounting media (Vector Laboratories). Images were acquired with a fluorescence microscope.

Preparations of total membranes and synaptosomes

Total membranes and synaptosomes from the hippocampus were prepared using sucrose/Percoll differential centrifugations, as described previously. Briefly, two mouse hippocampi were placed in ice-cold 0.32 M sucrose solution and instantly homogenized with a Teflon homogenizer. After centrifugation at 3000 g for 10 min, the supernatant was collected and divided to prepare synaptosomes or total membranes. To prepare total membranes, the supernatant was centrifuged at 28000 g for 60 min and the pellet was collected. To prepare synaptosomes, the supernatant was centrifuged at 14 000 g for 12 min and the pellet was resuspended in 1 ml of a solution of 45% (v/v) Percoll in Krebs solution. After centrifugation, the top layer (synaptosomal fraction) was incubated and washed with 1 ml ice-cold Krebs solution. The synaptosomes or total membranes were further resuspended in radioimmunoprecipitation assay buffer supplemented with a cocktail of protease inhibitors (Roche, Basel, Switzerland) for western blot analysis or in Krebs solution for immunocytochemical analysis.

Western blot analysis

Western blot analysis was carried out as described previously. Briefly, after determining the amount of protein, total membrane or synaptosomal samples were diluted with five volumes of sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer. These diluted samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer. The membrane protein was then transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, US). After blocking for 1 h at room temperature with 5% milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBS-T), the membranes were incubated overnight at 4 °C with a rabbit anti-A2AR antibody (1:500, 05-717; Millipore). After three washing, the membranes were incubated with horseradish peroxidase goat anti-mouse secondary antibody (1:1500; Pierce, Rockford, IL, USA) in TBS-T during 120 min at room temperature. After three washes, the membranes were incubated with Luminata Forte (Millipore) during 5 min and then analyzed with a VersaDoc 3000 (Bio-Rad, Amadora, Portugal) to quantify A2AR immunoreactivity as well as mCherry fluorescence. The membranes were then reprobed and normalized for α-tubulin (1:20 000; Sigma, St. Louis, MO, USA) immunoreactivity.

Immunocytochemistry of synaptosomes

The immunocytochemical analysis of hippocampal synaptosomes, to detect the presence of optoA2AR in glutamatergic terminals, was carried out as reported previously. The synaptosomes were placed onto coverslips coated with poly-β-lysine, fixed with 4% paraformaldehyde in PBS for 15 min and washed two times with PBS. The synaptosomes were permeabilized in PBS with 0.2% Triton X-100 for 10 min and then blocked for 1 h in PBS with 3% bovine serum albumin and 5% normal bovine serum. The synaptosomes were incubated overnight at 4 °C with either a mouse anti-A2AR antibody (1:500; 05-717; Millipore) or with a guinea-pig vesicular glutamate transporters type-1 antibody (1:2500; 1535304; Synaptic Systems, Göttingen, Germany), followed by incubation of donkey Alexa Fluor-488-conjugated anti-mouse IgG (alone) and goat Alexa Fluor-488-conjugated anti-guinea-pig IgG (1:1000; Molecular Probes). The colocalization of vesicular glutamate transporters type-1 or A2AR immunoreactivities with mCherry fluorescence was examined under a Zeiss Imager Z2 fluorescence microscope (Zeiss, Göttingen, Germany) and analyzed with ImageJ 1.37v software (NIH, Bethesda, MD, USA), as described previously.

RESULTS

Light activation of optoA2AR specifically recruits A2AR signaling in HEK293 cells

We engineered a light-activated chimeric protein able to recruit A2AR signaling, optoA2AR, by replacing the intracellular domains of rhodopsin with those of A2AR (Figure 1a). At 24 h after transfecting HEK293 cells with optoA2AR, we observed a single band with a 80 kDa molecular weight, expected for optoA2AR (Figure 1b), using an A2AR antibody targeting the third intracellular loop of A2AR. We also detected the red fluorescence of mCherry, included in the optoA2AR construct, largely restricted to the cell surface (Figure 1c), similar to that obtained using the A2AR antibody.

We activate the Gs/CAMP pathway as well as MAPK pathway in a Gαi-independent manner. Light stimulation of HEK293-optoA2AR cells (for 60 s) increased p-MAPK, in contrast with the weak p-MAPK immunoreactivity in light-stimulated cells transfected with the pcDNA3.1 vector (Figure 1d), which was confirmed by western blot (Figure 1e). Light stimulation of HEK293-optoA2AR cells also increased cAMP levels by twofold (immunoassay after 20 min), compared with non-stimulated HEK293-optoA2AR cells and with light-stimulated cells transfected with pcDNA3.1 (Figure 1f; P < 0.001, two-way ANOVA). Thus, optoA2AR specifically recruits the two parallel A2AR signaling pathways, namely Gs/CAMP and MAPK signaling, in HEK293 cells. Supporting the specificity of optoA2AR signaling, light stimulation of HEK293-optoA2AR cells induced CAMP and p-MAPK signaling (Figures 1e and f) but did not affect either cGMP (the...
rhodopsin transducing system; Figure 1g) or IP1 production (a degradation product of IP3, associated with Gq signaling; Figure 1h). Further reinforcing the selectivity of optoA2AR to trigger cAMP accumulation, a Ser400Ala point mutation in an A2AR phosphorylation site critical for A2AR-D2R receptor interaction,38 but not a Thr324Ala point mutation in an A2AR phosphorylation site critical for short-term desensitization,39 of the C terminals of optoA2AR abolished the light optoA2AR-induced cAMP accumulation (Figure 1i; **P < 0.01, Student’s t-test). Thus, optoA2AR signaling is specific and attributed to the unique amino-acid composition of its C terminus. Lastly, light stimulation of optoA2AR rapidly increased cAMP and p-MAPK levels in HEK293 cells within 1 min, peaking at 15–30 min and declining to basal level at 60–90 min (Figure 1j; P < 0.05, one-way ANOVA).

Light optoA2AR activation triggers an A2AR signaling identical to the pharmacological activation of endogenous A2AR both in HEK293 cells and mouse brain

To demonstrate the physiological relevance of optoA2AR signaling, we first compared cAMP levels and p-MAPK induced by either light activation of optoA2AR or CGS21680 (A2AR agonist) activation of wild-type A2AR in HEK293 cells. Light optoA2AR activation increased cAMP (Figure 2b; P < 0.001, two-way ANOVA) to levels similar to those triggered by CGS21680 (200 nM) in cells transfected with A2AR-mCherry (Figure 2a; P < 0.001, two-way ANOVA). Moreover, in cells co-transfected with A2AR-mCherry and optoA2AR, costimulation with light and CGS21680 produced additive effects on both cAMP level (Figure 2c; light, F(1,43) = 7.243, P < 0.01; CGS21680, F(3,43) = 32.674, P < 0.001; lightxCGS21680 interaction, F(3,45) = 0.336, P > 0.05, two-way ANOVA). Costimulation of light and CGS21680 in cells co-transfected with optoA2AR and wild-type A2AR produced an additive effect on cAMP levels (c; for light stimulation, F(1,43) = 7.243, P < 0.01; for CGS21680, F(3,43) = 32.674, P < 0.001; lightxCGS21680 interaction, F(3,45) = 0.336, P > 0.05, two-way ANOVA). *P < 0.05, **P < 0.01, two-way ANOVA, Bonferroni post hoc test. Following intra-accumbal injection, CGS21680 markedly induced phospho-mitogen-activated protein kinase (p-MAPK) expression (green) but not c-Fos expression around the injection site (d, lower panel). Following intrahippocampal injection, CGS21680 induced c-Fos expression (right) but not p-MAPK expression (left) around the injection site (e, upper panel). (f and g) Two weeks after intrahippocampal injection of AAV5-optoA2AR, light stimulation for 5 min induced c-Fos expression in optoA2AR-positive cells but not in cells transfected with AAV5-mCherry (3 fields per section, 3 sections per mouse, 3 mice per group). ***P < 0.001, Student’s t-test comparing the optoA2AR with the mCherry. Scale bar = 50 μm (d–g).

Figure 2. OptoA2AR and CGS21680 produced additive induction of cAMP in human embryonic kidney 293 (HEK293) cells and indistinguishable biased A2AR signaling in the nucleus accumbens (NAc) and hippocampus. (a–c) HEK293 cells were transfected with wild-type A2AR (a) or optoA2AR (b) or co-transfected with wild-type A2AR and optoA2AR (c). (a) At 24 h after transfection, cells transfected with wild-type A2AR were treated with the A2AR agonist CGS21680 (20, 100, 200 and 1000 nM) and displayed a concentration-dependent increase of cAMP levels. Light activation of optoA2AR-transfected HEK293 cells increased cAMP levels (b; for light, F(1,31) = 60.721, P < 0.001; for CGS21680, F(3,31) = 2.163, P > 0.05; lightxCGS21680 interaction, F(3,31) = 0.155, P > 0.05, two-way analysis of variance (ANOVA)) similar to that induced by 200 nM CGS21680 (a; for light, F(1,40) = 0, P > 0.05; for CGS21680, F(3,40) = 312.799, P < 0.001; lightxCGS21680 interaction, F(3,40) = 0.547, P > 0.05, two-way ANOVA). Costimulation of light and CGS21680 in cells co-transfected with optoA2AR and wild-type A2AR produced an additive effect on cAMP levels (c; for light stimulation, F(1,43) = 7.243, P < 0.01; for CGS21680, F(3,43) = 32.674, P < 0.001; lightxCGS21680 interaction, F(3,45) = 0.336, P > 0.05, two-way ANOVA). *P < 0.05, **P < 0.01, two-way ANOVA, Bonferroni post hoc test. Following intra-accumbal injection, CGS21680 markedly induced phospho-mitogen-activated protein kinase (p-MAPK) expression (green) but not c-Fos expression around the injection site (d, lower panel). Following intrahippocampal injection, CGS21680 induced c-Fos expression (right) but not p-MAPK expression (left) around the injection site (e, upper panel). (f and g) Two weeks after intrahippocampal injection of AAV5-optoA2AR, light stimulation for 5 min induced c-Fos expression in optoA2AR-positive cells but not in cells transfected with AAV5-mCherry (3 fields per section, 3 sections per mouse, 3 mice per group). ***P < 0.001, Student’s t-test comparing the optoA2AR with the mCherry. Scale bar = 50 μm (d–g).
stimulation in the hippocampus significantly increased c-Fos expression specifically in the optoA2AR-expressing cells under the cannula (Figure 2f), whereas optoA2AR activation in NAc markedly induced p-MAPK but not c-Fos (Figure 2g). This demonstrates a similarly biased A2AR signaling triggered by optoA2AR and CGS21680, that is, c-Fos in the hippocampus and p-MAPK in the NAc, supporting the ability of optoA2AR to mimic the endogenous A2AR signaling in the brain.

Targeted expression and light activation of optoA2AR in glutamatergic terminals of the hippocampus induced hippocampal long-term potentiation (LTP) in brain slices. (a) Representative western blot analysis showing that an antibody against the third intracellular loop of A2AR recognized two bands at 80 and 95 kDa in hippocampal synaptosomes as well as in total membranes from mice transfected with AAV-mCherry-optoA2AR (optoA2AR) but not from mice transfected with AAV-mCherry, compatible with the localization of optoA2AR in hippocampal synapses (n = 3). (b) Representative single nerve terminal immunocytochemistry identifying that vesicular glutamate transporter type-1 (vGluT1, a marker of glutamatergic terminals; green) and mCherry immunoreactivity (red) were found to be colocalized (arrows identifying yellow in ‘merged’) in hippocampal synaptosomes from mice transfected with AAV-mCherry-optoA2AR (optoA2AR), whereas this was not observed for mice transfected with AAV-mCherry (not shown) (n = 3). (c) Accordingly, light stimulation (3000 pulses of 50-ms duration each during 300 s) of slices from mice transfected with AAV-mCherry-optoA2AR, applied before a high-frequency train (100 Hz for 1 s), enhanced the amplitude of LTP compared with non-light-stimulated slices, measured as an increased slope of field excitatory postsynaptic potentials (fEPSP) recorded in the stratum radiatum of the CA1 area upon stimulation of the afferent Schaffer fibers (c), whereas light stimulation failed to modify LTP amplitude in mice transfected with AAV-mCherry (not shown). This essentially mimics the effect of the pharmacological activation of endogenous A2AR with the selective A2AR agonist CGS21680 (30 nM), in slices from mice transfected either with AAV-mCherry-optoA2AR (d) or with AAV-mCherry (e). Representative images (a and b) and data (mean ± s.e.m., c–e) are from n = 3 independent mice. *P < 0.05, Student’s t-test.

Figure 3. Targeted expression and light activation of optoA2AR in glutamatergic terminals of the hippocampus induced hippocampal long-term potentiation (LTP) in brain slices. (a) Representative western blot analysis showing that an antibody against the third intracellular loop of A2AR recognized two bands at 80 and 95 kDa in hippocampal synaptosomes as well as in total membranes from mice transfected with AAV-mCherry-optoA2AR (optoA2AR) but not from mice transfected with AAV-mCherry, compatible with the localization of optoA2AR in hippocampal synapses (n = 3). (b) Representative single nerve terminal immunocytochemistry identifying that vesicular glutamate transporter type-1 (vGluT1, a marker of glutamatergic terminals; green) and mCherry immunoreactivity (red) were found to be colocalized (arrows identifying yellow in ‘merged’) in hippocampal synaptosomes from mice transfected with AAV-mCherry-optoA2AR (optoA2AR), whereas this was not observed for mice transfected with AAV-mCherry (not shown) (n = 3). (c) Accordingly, light stimulation (3000 pulses of 50-ms duration each during 300 s) of slices from mice transfected with AAV-mCherry-optoA2AR, applied before a high-frequency train (100 Hz for 1 s), enhanced the amplitude of LTP compared with non-light-stimulated slices, measured as an increased slope of field excitatory postsynaptic potentials (fEPSP) recorded in the stratum radiatum of the CA1 area upon stimulation of the afferent Schaffer fibers (c), whereas light stimulation failed to modify LTP amplitude in mice transfected with AAV-mCherry (not shown). This essentially mimics the effect of the pharmacological activation of endogenous A2AR with the selective A2AR agonist CGS21680 (30 nM), in slices from mice transfected either with AAV-mCherry-optoA2AR (d) or with AAV-mCherry (e). Representative images (a and b) and data (mean ± s.e.m., c–e) are from n = 3 independent mice. *P < 0.05, Student’s t-test.

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glutamatergic nerve terminals, where endogenous A_{2A}R have been identified.

Additionally, we observed (Figure 3c) that the amplitude of LTP triggered by HFS was larger upon light stimulation (5 min before HFS) of hippocampal slices expressing optoA_{2A}R (197.3 ± 2.9% over baseline) than without light stimulation (137.7 ± 2.0% over baseline) (P = 0.05, Student’s t-test). By contrast, light stimulation did not modify LTP amplitude in hippocampal slices expressing only mCherry (138.9 ± 2.7% vs 142.5 ± 1.5% over baseline with or without light stimulation, respectively, n = 3; P > 0.05, Student’s t-test) (data not shown). This enhancement upon light-induced optoA_{2A}R activation was similar to the impact of a pharmacological activation of endogenous A_{2A}R with the A_{2A}R agonist, CGS21680 (30 nM) (Figure 3d), as well as in slices from mice transfected with AAV-mCherry (Figure 3e), as occurs in the wild-type animals. This shows that light activation of optoA_{2A}R can mimic an established physiological response operated by endogenous A_{2A}R in the hippocampus.

Optogenetic optoA_{2A}R activation in the hippocampus recruits CREB phosphorylation and impairs memory performance

Two weeks after the focal injection of AAV5-CaMKIIa promoter-driven-optoA_{2A}R-mCherry (Figure 4a), light stimulation in the dorsal hippocampus significantly increased the levels of p-CREB ( Figures 4c and d; P < 0.001, Student’s t-test) specifically in the optoA_{2A}R-expressing neurons underneath the cannula, consistent with the A_{2A}R-Gs-cAMP pathway as the major A_{2A}R signaling pathway in the hippocampus.

Similar to p-CREB recruitment, light stimulation significantly elevated c-Fos in the optoA_{2A}R-expressing neurons in hippocampus (Figure 2f; P < 0.001, Student’s t-test), whereas it did not induce p-MAPK (Figures 4b and d). Thus, in hippocampal neurons, light optoA_{2A}R activation preferentially stimulates the cAMP-PKA pathway, leading to p-CREB and c-Fos expression, without significant effect on the p-MAPK pathway.

To address the central question whether A_{2A}R activation in the hippocampus is sufficient to impair memory performance, we tested if triggering hippocampal optoA_{2A}R signaling affected spatial reference memory performance using a two-visit version of the Y-maze test. Light optoA_{2A}R activation in the hippocampus during the 5-min testing period reduced about twofold the time travelled (f; AAV vector (F(1,41) = 0.45, P > 0.05, two-way analysis of variance (ANOVA)) during on the 5-min ‘Light-On’ period, compared with that of the control. Green arrow: colocalization of optoA_{2A}R with p-CREB; red arrow: p-CREB expression only. Scale bar = 50 μm.

**Figure 4.** Light activation of hippocampal optoA_{2A}R triggers CREB phosphorylation and memory impairment. (a) Upper panel: Schematic illustration of transplanted cannula targeting hippocampus and expression of mCherry (optoA_{2A}R) under CaMKIIa promoter in the hippocampus after focal injection of AAV5-optoA_{2A}R-mCherry (AP, -2.2 mm; ML, ±1.5 mm; DV, +2.3 mm). Lower panel: colocalization of A_{2A}R immunostaining (green) with mCherry-expressing (red) in optoA_{2A}R-expressing cells. (b and c) Light stimulation of optoA_{2A}R in the hippocampus for 5 min induced phospho-CREB (p-CREB) (b and d) but not phospho-mitogen-activated protein kinase (p-MAPK) (c and d) (P < 0.001, Student’s t-test) in optoA_{2A}R-positive but not in cells transfected with AAV5-mCherry (3 fields per section, 3 sections per mouse, 3 mice per group). (e) Light stimulation of optoA_{2A}R signaling in the hippocampus during the retrieval phase impaired spatial recognition memory with decreased time in the novel arm (%P) < 0.001, Student’s t-test) of the Y-maze but had no effect on total distance travelled (f; AAV vector (F(1,41) = 0.45, P > 0.05, two-way analysis of variance (ANOVA)) during on the 5-min ‘Light-On’ period, compared with that of the control. Green arrow: colocalization of optoA_{2A}R with p-CREB; red arrow: p-CREB expression only. Scale bar = 50 μm.

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Figure 5. Light activation of accumbal optoA2AR triggered phospho-mitogen-activated protein kinase (p-MAPK) phosphorylation and locomotor response. (a) Top left: Schematic illustration and mCherry (optoA2AR) under CaMKIIα promoter in nucleus accumbens (NAc) after focal injection of AAV5-optoA2AR-mCherry in the NAc (AP, +1.1 mm; ML, ±1.4 mm; DV, +4.5 mm). Top left: Schematic illustration and location of mCherry in the core and shell of NAc. Top right: A2AR immunostaining (green) localized in mCherry-expressing (red) optoA2AR-positive cells. Bottom left: optoA2AR-mCherry were colocalized in NeuN-positive (green) but not GFAP-positive cells. (b) Expression of optoA2AR in enkephalin (ENK)-positive and substance P (SP)-positive medium-sized spiny neurons. (c and d) Light induction of phospho-CREB (p-CREB) (c) and p-MAPK (d) in the NAc at 2 weeks after intra-NAc injection of AAV5-optoA2AR (right) or AAV5-mCherry (left). Light stimulation produced scattered expression of p-CREB but triggered a robust induction of p-MAPK with mCherry-expressing in optoA2AR-positive cells (right panels). (e) Quantitative analysis of light induction of p-CREB and p-MAPK in optoA2AR-positive cells but not in cells transfected with AAV5-mCherry. p-CREB/p-MAPK-positive cell accounts were obtained from 3 fields per section, 3 sections per mouse and 3 mice per group. ***P < 0.001, Student's t-test comparing the optoA2AR with the mCherry. (f and g) Light stimulation of optoA2AR in the NAc increased the total distance travelled at the acquisition phase (g; for AAV vector, F(1, 44) = 10.11, P < 0.003; for behavioral phase, F(1, 44) = 25.08, P < 0.001; AAV vectorxbehavioral phase, F(1, 44) = 18.68, P < 0.001, two-way analysis of variance (ANOVA); ***P < 0.001, **P < 0.001, Bonferroni post hoc test, comparing optoA2AR with mCherry) but had no effect time spent in the novel arm during the 5-min 'Light-On' period, compared with that of the mCherry (f; P = 0.276, Student's t-test). Scale bar = 50 μm. Green arrow: colocalization of optoA2AR with p-CREB; red arrow: p-CREB expression only; white arrow: colocalization of optoA2AR with ENK, SP, p-CREB or phospho-MAPK; green arrow: ENK, SP, p-CREB or phospho-MAPK only; red arrow: mCherry expression only.
(p-CREB and p-MAPK). As GPCR can produce a biased signaling simply due to different receptor levels, we used the same CaMKIIa promoter to drive similar levels of optoA2AR expression in hippocampal and striatal neurons,40,41 to eliminate different optoA2AR expression levels as a possible cause of a biased A2AR signaling in these two forebrain regions.32 OptoA2AR was selectively expressed in accumbal neurons (colocalized with Neu+ neurons but not with GFAP+ astrocytes) in the core of NAc (Figure 5a). Using enkephalin (ENK) and substance P immunostaining to identify the indirect and direct pathway neurons,33 we found that optoA2AR was expressed in both ENK- (52%) and SP-containing (43%) neurons in NAc (Figure 5b). Importantly, light activation of optoA2AR in NAc for 5 min markedly increased p-MAPK (Figures 5d and e; P < 0.001, Student’s t-test) with little induction of p-CREB and c-Fos (Figures 5c, e and 2g). Thus, the activation of A2AR signaling by optoA2AR in NAc preferably involved the MAPK pathway rather than the cAMP-PKA-mediated c-Fos/CREB pathway.

In parallel with the preferential activation of p-MAPK signaling, light optoA2AR activation in NAc for 5 min did not affect memory performance in the modified Y-maze test (Figure 5f; P = 0.276, Student’s t-test), but robustly increased locomotor activity (83% increase of travelled distance in the Y-maze, Figure 5g, and in the open-field test) (for AAV vector×behavioral phase, F(1,45) = 18.68, P < 0.001, two-way ANOVA). The observed motor stimulant effect resulting from optoA2AR activation in the NAc, instead of a motor depression observed upon accumbal administration of CGS21680,44,45 was expected in view of the expression of endogenous A2AR in both striatopallidal and striatonigral neurons using the CaMKIIa promoter41,42 (rather than the selective expression of endogenous A2AR in striatopallidal neurons prompted by the A2AR promoter46). This pitfall was however essential to circumvent the confounding effect of a 20-fold differential expression of A2AR in these two brain regions as a possible cause for the biased A2AR signaling. Overall, the present findings show that A2AR trigger a biased A2AR signaling in different forebrain regions (cAMP in the hippocampus and p-MAPK in the NAc) in parallel with an impact on distinct behaviors (memory in the hippocampus and locomotion in NAc).

**DISCUSSION**

The development and validation of the optoA2AR approach to mimic endogenous A2AR signaling allowed the novel conclusion that the recruitment of A2AR signaling in the dorsal hippocampus is sufficient to trigger a selective memory deficit. This is in accordance with the imbalance of the local extracellular adenosine levels21 and upregulation of A2AR in animal models of aging,22 sporadic dementia11 and AD,10 as well as in the human AD brain,38 namely in hippocampal nerve terminals,12,13 a situation that was mimicked by hippocampal optoA2AR expression under the control of the CaMKIIa promoter. Indeed, optoA2AR was detected in hippocampal synaptosomes, namely in glutamatergic synapses where endogenous A2AR are identified and upregulated upon aging and neurodegeneration. This provides an anatomical basis for optoA2AR signaling of endogenous A2AR activity and memory performance. Indeed, the light activation of optoA2AR in hippocampal slices mimicked a well-established physiological response operated by endogenous A2AR, the control of hippocampal LTP.39 Furthermore, optoA2AR activation in the hippocampus triggers CREB phosphorylation and impairs memory performance. These findings are consistent with the canonical cAMP/PKA pathway activated by hippocampal A2AR42 and with the established role of CREB phosphorylation controlling synaptic plasticity and long-term memory39 through neuronal excitability and transcription, and with specific deficits of memory retrieval observed in mice expressing a time-controlled active CREB variant.33 This ability of hippocampal optoA2AR activation in glutamate synapses to control memory dysfunction and its purported neurophysiological correlate LTP, decisively strengthens the relation between A2AR and memory performance that had so far largely relied on the demonstration that A2AR blockade alleviated memory dysfunction.8,9,12,14,15 Furthermore, this ability to place A2AR functioning as a sufficient factor to imbalance memory bolsters the rationale to probe the therapeutic effectiveness of A2AR antagonists to manage memory impairments22,23 This notion is further warranted by the striking convergence of epidemiological16–18 and animal19–20 evidence supporting the therapeutic benefit of caffeine and A2AR antagonists to improve cognition. This aim should be facilitated by the safety profile of A2AR antagonists, tested in over 3000 parkinsonian patients.21–23

The design of optoA2AR also allowed identifying a critical role solely attributable to the intracellular domains of A2AR to dictate the biased A2AR signaling and function in neurons of different brain regions. Contrary to the widely accepted view that ligand-receptor interactions are the molecular basis directing the biased GPCR signaling, the distinct molecular and behavioral responses obtained upon optoA2AR activation in different brain regions show that they are only dependent on an intracellular mechanism probably related with the differential association with different GIPs in different cell types. In fact, the cell-specific expression of intracellular GIPs provides a rich molecular resource26 whereby A2AR signaling in the brain is specifically wired according to the needs of each cell type. In particular, the long and flexible A2AR C terminus29–31 contains several consensus sites (e.g. YXXG) required for MAPK activation31 interaction with BDNF receptors (TrkB),52 with FGF,53 p53,54,55 with a large set of downstream signaling effectors such as G proteins, GPCR kinases, arrestins and with at least six GIPs (actinin, calmodulin, NcAb2, translin-associated protein X, ARNO/cytohesin-2, ubiquitin-specific protease-4).29 Thus, targeting A2AR intracellular domains offers an additional layer of selectivity to manipulate A2AR signaling that is not attainable only by the ligand–receptor interaction. Thus, selectively targeting A2AR intracellular domains and their interacting GIPs in specific brain regions emerges as a novel strategy to obtain therapeutic effects with minimal side effects, as achieved with transmembrane peptides to disrupt specifically the intracellular interaction between NMDA receptors and PSD9556,57 and between 5-HT2c-PENT.58 If the critical interaction between intracellular domains of A2AR and GIPs are general features of GPCRs, the ‘optoGPCR’ approach targeting intracellular domains of GPCRs may represent a novel drug discovery strategy for the largest protein superfamily in the human genome.

The significance of these novel insights is decisively strengthened by the demonstrated specificity and rapid induction of the optoA2AR signaling. The specificity of optoA2AR signaling is supported by the selective optogenetic induction of cAMP and MAPK signaling without affecting cGMP (rhodopsin) and IP3 (G αq) signaling and by the mutational analysis demonstrating that optoA2AR signaling is specifically attributed to the unique amino acid composition of the A2AR C terminus. Moreover, the comparable activation of A2AR signaling in HEK293 cells and the indistinguishable pattern of the biased A2AR signaling (length of the NAc and the hippocampus, as well as the similar enhancement of hippocampal LTP triggered by optoA2AR and CGS21680 supports that optoA2AR signaling largely captures the physiological function of the native A2AR. Different from opsin-based optogenetics, opto-A2AR signals through GPCR signaling allows a control of intracellular A2AR signaling by light, which we now report to involve a rapid induction, consistent with similar rapid physiological response (T on/off = 1 s) of other GPCR light-activated chimera. Thus, the temporal and spatial control of specific A2AR signaling afforded by optoA2AR in freely behaving animals paves the way to probe the role of A2AR in defined forebrain circuits responsible for behaviors ranging from motor control, fear, addiction, mood or decision making.59–61
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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REFERENCES
1 van Boxtel MP, Schmitt JA, Bosma H, Jolles J. The effects of habitual caffeine use on cognitive change: a longitudinal perspective. Pharmacol Biochem Behav 2003; 75: 921–927.
2 Hameleers PA, Van Boxtel MP, Hogervorst E, Riedel WJ, Houx PJ, Buntinx F, et al. Habitual caffeine consumption and its relation to memory, attention, planning capacity and psychomotor performance across multiple age groups. Hum Psychopharmacol 2000; 15: 573–581.
3 Lindsay J, Launin D, Vermeulen R, Hebert R, Hellswell B, Hill GB, et al. Risk factors for Alzheimer’s disease: a prospective analysis from the Canadian Study of Health and Aging. Am J Epidemiol 2002; 156: 445–453.
4 van Gelder BM, Buijsse B, Tijhuis M, Kalmijn S, Giampaoli S, Nissinen A, et al. Multifactorial risk of type 2 diabetes-induced memory impairment and synaptotoxicity in the hippocampus of aged rats. J Neurochem 2009; 23: 307–322.
5 Ritchie K, Carriere I, de Mendonca A, Portet F, Dartigues JF, Rouaud O, et al. The neuroprotective effects of caffeine: a prospective population study (the three City Study). Neurology 2007; 69: 536–545.
6 Gelber RP, Petrovitch H, Masaki KH, Ross GW, White LR. Coffee intake in midlife and risk of dementia and its neuropathological correlates. J Alzheimers Dis 2011; 23: 607–615.
7 Eskelin MN, Ngandu T, Tuomainen JT, Soininen H, Kivipelto M. Midlife coffee and tea drinking and the risk of late-life dementia: a population-based CAIDE study. J Alzheimers Dis 2009; 16: 85–91.
8 Dallfågna OP, Fett P, Gomes MW, Souza DO, Cunha RA, Lara DR. Caffeine and adenosine A2A receptor antagonists prevent beta-amyloid (25–35)-induced cognitive deficits in mice. Exp Neurol 2007; 203: 241–245.
9 Cunha GM, Canas PM, Melo CS, Hockemeyer J, Muller CE, Oliveira CR, et al. Adenosine A2A receptor blockade prevents memory dysfunction caused by beta-amyloid peptides but not by scopolamine or MK-801. Am J Physiol Regul Integr Comp Physiol 2007; 293: R1601–R1612.
10 Arendash GW, Schleif W, Rezai-Zadeh K, Jackson EK, Zacharia LC, Cracchiolo JR, et al. Adenosine A2A receptor blockade prevents synaptotoxicity and memory dysfunction caused by beta-amyloid peptides but not by scopolamine or MK-801. Alzheimer's disease: a prospective analysis from the Canadian Study of Health and Aging. Am J Epidemiol 2002; 156: 445–453.
11 Wei CJ, Singer P, Coelho I, Boison D, Feldon J, Yee BK, et al. Selective inactivation of adenosine A2A receptors in striatal neurones enhances working memory and reversal learning. Learn Mem 2011; 18: 459–474.
12 Yu C, Gupta J, Chen JF, Yin H. Genetic deletion of A2A adenosine receptors in the striatum selectively impair habit formation. J Neurosci 2009; 29: 15100–15103.
13 Wei CJ, Augusto E, Gomes CA, Singer P, Wang Y, Boison D, et al. Regulation of fear responses by striatal and extrastriatal adenosine A2A receptors in forebrain. Biol Psychiatry 2013; 75: 855–863.
14 Kadowaki Horita T, Kobayashi M, Mori A, Jennen P, Kanda T. Effects of the adenosine A2A antagonist istradefylline on cognitive performance in rats with a 6-OHDA lesion in prefrontal cortex. Psychopharmacology (Berl) 2013; 230: 345–352.
15 Cunha RA, Almeida T, Ribeiro JA. Parallel modification of adenosine extracellular r metabolism and modulatory action in the hippocampus of aged rats. J Neurochem 2001; 76: 372–382.
16 Chen JF, Etzschig HK, Fredholm BB. Adenosine receptors as drug targets—what are the challenges? Nat Rev Drug Discov 2013; 12: 265–286.
17 Cunha RA, Agostinho PM. Chronic caffeine consumption prevents memory disturbance in different animal models of memory decline. J Alzheimers Dis 2010; 20: 595–116.
18 Chen JF, Sonsalla PK, Pedata F, Melani A, Domenici MR, Popoli P, et al. Adenosine A2A receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and ‘fine tuning’ modulation. Prog Neurobiol 2007; 83: 310–331.
19 Fredholm BB, Chen Y, Franco R, Sittkovsky M. Aspects of the general biology of adenosine A2A signaling. Prog Neurobiol 2007; 83: 263–276.
20 Shen HY, Canas PM, Garcia-Sanz P, Lan JQ, Boison D, Moratalla R, et al. Adenosine A2A receptors in striatal glutamatergic terminals and GABAergic neurons oppositely modulate psychostimulant action and DARPP-32 phosphorylation. PLoS One 2013; 8: e69092.
21 Shen HY, Coelho JE, Ohtsuka N, Canas PM, Day YJ, Huang QY, et al. A critical role of the adenosine A2A receptor in extrastriatal neurones in modulating locomotor activity as revealed by opposite phenotypes of striatum and forebrain A2A receptor knock-outs. J Neurosci 2008; 28: 2970–2975.
22 Ciruela F, Casado V, Rodrigues RJ, Lujan R, Burgueno J, Canals M, et al. Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1–A2A receptor heteromers. J Neurosci 2006; 26: 2080–2087.
23 Kruegerleber S, Gandtner F, Freismuth M. From cradle to twilight: the carboxyl terminus directs the fate of the A2A-adenosine receptor. Biochim Biophys Acta 2011; 1808: 1350–1357.
24 Mondelli S, Kelly E. Adenosine receptor desensitization and trafficking. Biochim Biophys Acta 2011; 1808: 1319–1328.
25 Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K. Millisecond-timescale genetically targeted optical control of neural activity. Nat Neurosci 2005; 8: 1263–1268.
26 Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, et al. A2A(2) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. J Neurosci 1999; 19: 9192–9200.
27 Costenla AR, Dogenes MJ, Canas PM, Rodrigues RJ, Nogueira C, Maroco J, et al. Enhanced role of adenosine A2A receptors in the modulation of LTP in the rat hippocampus upon ageing. The European Journal of Neuroscience 2011; 34: 12–21.
28 Cognato GP, Agostinho PM, Hockemeyer J, Muller CE, Souza DO, Cunha RA, et al. Caffeine and an adenosine A2A receptor antagonist prevent memory impairment and synaptic plasticity in adult rats triggered by a convulsive episode in early life. Journal of Neurochemistry 2010; 112: 453–462.
29 Rebola N, Canas PM, Oliveira CR, Cunha RA. Different synaptic and subnuclear localization of adenosine A2A receptors in the hippocampus and striatum of the rat. Neuroscience 2005; 132: 893–903.
30 Rebola N, Rodrigues RJ, Lopes LV, Richardson PJ, Oliveira CR, Cunha RA, Adenosine A1 and A2A receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus. Neuroscience 2005; 133: 79–83.
31 Rodrigues RJ, Canas PM, Oliveira CR, Cunha RA. Modification of adenosine modulation of acetylcholine release in the hippocampus of aged rats. Neurobiology of Aging 2008; 29: 1597–1601.
32 Boronzo-Escuela DO, Romero-Fernandez W, Tarakanov AO, Gomez-Soler M, Coelles F, Marcellino D, et al. Characterization of the A2A-D2R interface: focus on the role of the C-terminal tail and the transmembrane helices. Biochem Biophys Res Commun 2010; 402: 801–807.
33 Viozca J, Mallert G, Bourcouladze R, Benito E, Vrionska S, Kandel ER, et al. Chronic enhancement of CREB activity in the hippocampus interferes with the retrieval of spatial information. Learn Mem 2009; 16: 198–209.
34 Brit JP, Benalamoud F, McDevitt RA, Stuber GD, Wise RA, Bonci A. Synaptic and behavioral profile of multiple glutamatergic inputs to the nucleus accumbens. Neuron 2012; 76: 790–803.
41 Chuhma N, Tanaka KF, Hen R, Rayport S. Functional connectome of the striatal medium spiny neuron. J Neurosci 2011; 31: 1183–1192.
42 Kenakin T, Christopoulos A. Signalling bias in new drug discovery: detection, quantification and therapeutic impact. Nat Rev Drug Discov 2013; 12: 205–216.
43 Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma FJ Jr, et al. D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. Science 1990; 250: 1429–1432.
44 Hauber W, Munkle M. Motor depressive effects mediated by dopamine D2 and adenosine A2A receptors in the nucleus accumbens and the caudate–putamen. Eur J Pharmacol 1997; 323: 127–131.
45 Barraco RA, Martens KA, Parizon M, Normile HJ. Role of adenosine A2A receptors in the nucleus accumbens. Prog Neuropsychopharmacol Biol Psychiatry 1994; 18: 545–553.
46 Svenningsson P, Le Moine C, Fisone G, Fredholm BB. Distribution, biochemistry and function of striatal adenosine A2A receptors. Prog Neurobiol 1999; 59: 355–396.
47 Canas PM, Duarte JM, Rodrigues RJ, Kofalvi A, Cunha RA. Modulation of pre- and postsynaptic modulation systems in the hippocampus. Neurobiol Aging 2009; 30: 1877–1884.
48 Albasanz JL, Perez S, Barrachina M, Ferrer I, Martin M. Up-regulation of adenosine A2A receptors in the frontal cortex in Alzheimer’s disease. Brain Pathol 2008; 18: 211–219.
49 Benito E, Barco A. CREB’s control of intrinsic and synaptic plasticity: implications for CREB-dependent memory models. Trends Neurosci 2010; 33: 230–240.
50 Bockeart J, Perroy J, Bécamel C, Marin P, Fagni L. GPCR interacting proteins (GIPs) in the nervous system: roles in physiology and pathologies. Annu Rev Pharmacol Toxicol 2010; 50: 89–109.
51 Gsandtner I, Charalambous C, Stefan E, Ogris E, Freissmuth M, Zezula J. Hetero-trimeric G protein-independent signaling of a G protein-coupled receptor. Direct binding of ARNO/cytohesin-2 to the carboxyl terminus of the A2A adenosine receptor is necessary for sustained activation of the ERK/MAP kinase pathway. J Biol Chem 2005; 280: 31898–31905.
52 Rajagopal R, Chen ZY, Lee FS, Chao MV. Transactivation of Trk neurotrophin receptors by G-protein-coupled receptor ligands occurs on intracellular membranes. J Neurosci 2004; 24: 6650–6658.
53 Flajolet M, Wang Z, Futter M, Shen W, Nuangchamnong N, Bendor J, et al. FGF acts as a co-transmitter through adenosine A2A receptor to regulate synaptic plasticity. Nat Neurosci 2008; 11: 1402–1409.
54 Sun CN, Cheng HC, Chou JL, Lee SY, Lin YW, Lai HL, et al. Rescue of p53 blockage by the A2A adenosine receptor via a novel interacting protein, translin-associated protein X. Mol Pharmacol 2006; 70: 454–466.
55 Sun CN, Chuang HC, Wang YJ, Chen SY, Cheng YY, Lee CF, et al. The A2A adenosine receptor rescues neuritogenesis impaired by p53 blockage via KIF2A, a kinesin family member. Dev Neurobiol 2010; 70: 604–621.
56 Aarts M, Liu Y, Liu L, Bessho S, Arundine M, Gurd JW, et al. Treatment of ischemic brain damage by perturbing NMDA receptor–PSD-95 protein interactions. Science 2002; 298: 846–850.
57 Cook DJ, Teves L, Tymianski M. Treatment of stroke with a PSD-95 inhibitor in the gyrencephalic primate brain. Nature 2012; 483: 213–217.
58 Ji SP, Zhang Y, Van Cleemput J, Jiang W, Liao M, Li L, et al. Disruption of PTEN coupling with 5-HT2C receptors suppresses behavioral responses induced by drugs of abuse. Nat Med 2006; 12: 324–329.
59 Wei CJ, Li W, Chen JF. Normal and abnormal functions of adenosine receptors in the central nervous system revealed by genetic knockout studies. Biochim Biophys Acta 2011; 1808: 1358–1379.
60 Gomes CV, Kaster MP, Tome AR, Agostinho PM, Cunha RA. Adenosine receptors and brain diseases: neuroprotection and neurodegeneration. Biochim Biophys Acta 2011; 1808: 1380–1399.
61 Dellu F, Fauchey V, Le Maal M, Simon H. Extension of a new two-trial memory task in the rat: influence of environmental context on recognition processes. Neurobiol Learn Mem 1997; 67: 112–120.