Behavioral/Cognitive

Ecto-5′-Nucleotidase (CD73)-Mediated Formation of Adenosine Is Critical for the Striatal Adenosine A_{2A} Receptor Functions

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Adenosine is a neuromodulator acting through inhibitory A_1 receptors (A_1Rs) and facilitatory A_{2A}Rs, which have similar affinities for adenosine. It has been shown that the activity of intracellular adenosine kinase preferentially controls the activation of A_1Rs, but the source of the adenosine activating A_{2A}Rs is unknown. We now show that ecto-5′-nucleotidase (CD73), the major enzyme able to convert extracellular AMP into adenosine, colocalizes with A_{2A}Rs in the basal ganglia. In addition to astrocytes, striatal CD73 is prominently localized to postsynaptic sites. Notably, CD73 coimmunoprecipitated with A_{2A}Rs and proximity ligation assays confirmed the close proximity of CD73 and A_{2A}Rs in the striatum. Accordingly, the cAMP formation in synaptosomes as well as the hypolocomotion induced by a novel A_{2A}R prodrug that requires CD73 metabolization to activate A_{2A}Rs were observed in wild-type mice, but not in CD73 knock-out (KO) mice or A_{2A}R KO mice. Moreover, CD73 KO mice displayed increased working memory performance and a blunted amphetamine-induced sensitization, mimicking the phenotype of global or forebrain-A_{2A}R KO mice, as well as upon pharmacological A_{2A}R blockade. These results show that CD73-mediated formation of extracellular adenosine is responsible for the activation of striatal A_{2A}R function. This study points to CD73 as a new target that can fine-tune A_{2A}R activity, and a novel therapeutic target to manipulate A_{2A}R-mediated control of striatal function and neurodegeneration.

Introduction

Adenosine is a neuromodulator that fine-tunes brain neurotransmission mainly acting through inhibitory A_1 receptors (A_1Rs) and facilitatory A_{2A}Rs (Fredholm et al., 2005). A_1Rs are abundantly expressed throughout the brain, controlling synaptic transmission (Dunwiddie and Masino, 2001). A_1R activation depends on the tissue workload (Cunha, 2001a), and adenosine kinase activity is a key regulator of endogenous adenosine activating A_1Rs (Boison, 2011). In accordance with their inhibitory role curtailing excitatory transmission, bolstering A_1R activation through inhibition of adenosine kinase affords neuroprotection against brain damage involving glutamate excitotoxicity (Fredholm et al., 2005), namely upon epilepsy and brain ischemia (Boison, 2006). Importantly, the manipulation of the metabolic pathways associated with A_1R activation is more promising than the direct A_1R activation to control neurodegeneration, since the former locally enhances adenosine where activity is disrupted, whereas the latter also activates peripheral A_1R causing marked cardiovascular effects (Cunha, 2005).

The brain distribution of A_{2A}Rs is different from that of A_1Rs: A_{2A}Rs are most abundant in the basal ganglia (Schiffmann et al., 1991), but are also present at lower density throughout the brain (Rosin et al., 2003). Like A_1Rs, A_{2A}Rs are mostly located at synapses (Rebola et al., 2003, 2005), but they fulfill different roles. Thus, A_{2A}Rs are selectively engaged to assist the implementation of synaptic plastic changes in excitatory synapses (Cunha, 2008), by facilitating NMDA receptor-mediated responses (Rebola et al., 2008), by increasing glutamate release (Rodrigues et al., 2005), and by desensitizing presynaptic inhibitory modulation of systems like A_1Rs (Lopes et al., 2002; Ciruela et al., 2006) or cannabinoid CB_1Rs (Martire et al., 2011). Therefore, A_{2A}Rs play a key role in modulating the plasticity of neuronal circuits, such as upon learning and memory (Zhou et al., 2009; Wei et al., 2011)

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or drug addiction (Chen et al., 2003). Notably, neurodegenerative conditions are accompanied by an upregulation of A_{2A}Rs (Cunha, 2005), justifying that A_{2A}R blockade controls the burden of Parkinson's (Chen et al., 2001) or Alzheimer's disease (Canas et al., 2009).

The source of the adenosine activating A_{2A}Rs is poorly characterized. We have previously shown that different sources of adenosine activate A_1Rs and A_{2A}Rs (Cunha et al., 1996a) and that A_{2A}Rs are selectively activated upon extracellular catabolism by ecto-nucleotidases of ATP (Cunha et al., 1996a; Rebola et al., 2008). We have also shown that the ATP-derived formation of adenosine by ecto-nucleotidases is limited and controlled by ecto-5′-nucleotidase (CD73) activity (Cunha, 2001b), the only enzyme able to dephosphorylate extracellular AMP into adenosine in the brain (Lovatt et al., 2012). In agreement with this proposed functional association between CD73 and A_{2A}Rs, CD73 activity displays a brain distribution similar to A_{2A}Rs, both being higher in the basal ganglia (Langer et al., 2008).

Using mice deficient in either CD73 or A_{2A}Rs, coupled with a novel A_{2A}R agonist prodrug requiring a CD73-mediated activation and a proximity ligation assay (PLA), we now explored whether CD73 and A_{2A}Rs are colocalized and physically associated in the striatal neurons, and whether CD73 provides the particular pool of extracellular adenosine selectively responsible for activating striatal A_{2A}Rs.

Materials and Methods

Animals. Approval from the Institutional Animal Care and Use Committee at Boston University School of Medicine and the Portuguese Veterinarian Office was granted for all experiments conducted in Boston and Coimbra, respectively. They adhered to the NIH *Guide for the Care and Use of Laboratory Animals*, the Portuguese Law and Ordinance on Animal Protection, and European Council Directive 86/609/EEC. The knock-out (KO) mice used, both with a C57BL/6 genetic background, were previously characterized, namely global-CD73 KO (CD73 KO) (Thompson et al., 2004), as well as the global-A_{2A}R KO mice (A_{2A}R KO) (Chen et al., 1999). In all experiments, male and female adult (2–3 months old) mice were used.

Separation of total membranes. Mice were killed by decapitation after deep anesthesia with isoflurane, and the brain tissues were homogenized in sucrose (0.32 M) solution containing 1 mM EDTA, 10 mM HEPES, 1 mg/ml bovine serum albumin (BSA; Sigma), pH 7.4 at 4°C. The homogenates were centrifuged at 3000 × *g* for 10 min at 4°C, and the supernatants were then centrifuged at 14,000 × *g* for 10 min at 4°C. The pellets were washed in Krebs-HEPES-Ringer (KHR) solution containing 140 mM NaCl, 1 mM EDTA, 10 mM HEPES, 5 mM KCl, and 5 mM glucose, pH 7.4 at 4°C, and were further centrifuged at 14,000 × *g* for 10 min at 4°C. The pellets were either resuspended in the incubation buffer for binding studies or in radioimmunoprecipitation assay (RIPA) buffer for Western blot analysis.

Purification of synaptosomes and gliosomes. After the homogenization of the brain tissue, synaptosomes and gliosomes were obtained using a discontinuous Percoll gradient (2, 6, 15, and 23% v/v of Percoll in a medium containing 0.32 M sucrose and 1 mM EDTA, pH 7.4), as previously described (Matos et al., 2012a). The mixture was centrifuged at 31,000 × *g* for 5 min at 4°C with braking speed set down to 0 after reaching 1500 × *g* (Dunkley et al., 2008). The layers between 2% and 6% of Percoll (gliosomal fraction) and between 15% and 23% of Percoll (presynaptosomal fraction) were collected, washed in 10 ml of HEPES-buffered medium containing 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4, and further centrifuged at 22,000 × *g* for 15 min at 4°C to remove myelin components and postsynaptic material from the gliosomal and synaptosomal fractions, respectively. Both fractions were resuspended in RIPA buffer for Western blot analysis.

Separation of presynaptic, postsynaptic, and extrasynaptic fractions. The separation of the presynaptic active zone, postsynaptic density and nonsynaptic fractions from nerve terminals was performed by combining solubilization steps and changes in pH, as previously described (Rebola et al., 2005). Briefly, a solution with sucrose (1.25 M) and $CaCl_2$ (0.1 mM) was gently added to the tissue homogenates under agitation. Another sucrose solution (1 M) containing 0.1 mM $CaCl_2$ was gently stratified over the homogenate, followed by centrifugation (100,000 × *g* for 3 h at 4°C) to separate nuclei and debris (pellet), myelin (top layer) and the synaptosomes (interface between 1.25 and 1 M of sucrose), which were diluted 1:10 in sucrose solution (0.32 M) containing 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, and centrifuged (15,000 × *g* for 30 min at 4°C). The pellet (synaptosomes) was diluted 1:10 in cold 0.1 mM $CaCl_2$ and an equal volume of 2× solubilization buffer (2% Triton X-100, 40 mM Tris, pH 6.0) was added to the suspension. The membranes were incubated for 30 min on ice with mild agitation, and the insoluble material (synaptic junctions) was pelleted (40,000 × *g* for 30 min at 4°C). The supernatant (extrasynaptic fraction) was decanted and proteins were precipitated with six volumes of acetone at −20°C and recovered by centrifugation (18,000 × *g* for 30 min at −15°C). The synaptic junctions pellet was washed in the solubilization buffer, pH 6.0, and resuspended in 10 volumes of a second solubilization buffer (1% Triton X-100, 20 mM Tris but at pH 8.0). This increase in pH allows the dissociation of the extracellular matrix that maintains the presynaptic active zone tightly bound to the postsynaptic density (Phillips et al., 2001). Hence, the active zone is solubilized, whereas the postsynaptic density is essentially preserved because the amount of detergent is not enough for its solubilization (Phillips et al., 2001). After incubation for 30 min on ice with mild agitation, the mixture was centrifuged and the supernatant (presynaptic fraction) processed as described for the extrasynaptic fraction, whereas the final insoluble pellet corresponds to the postsynaptic fraction. The samples were resuspended in RIPA buffer for Western blot analysis.

Co-immunoprecipitation. Co-immunoprecipitation (Co-IP) was performed as previously described (Ciruela et al., 2001). Briefly, total membranes from the striatum (1 mg) were prepared as described above, washed in PBS (140 mM NaCl, 3 mM KCl, 20 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), and centrifuged at 14,000 × *g* for 10 min at 4°C. The pellets were either resuspended in the immunoprecipitation buffer (IPB; containing 20 mM Tris, pH 7.0, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 μM okadaic acid, 0.1 mM PMSF, and 1:1000 protease inhibitor cocktail) with 1% Triton X-100, sonicated for 30 s on ice, and further spun down for 10 min to remove insoluble materials. A sample was collected to determine the protein concentration using the bicinchoninic acid (BCA) assay (Thermo Scientific), another was stored at −20°C as input (positive control), and the rest of the sample was processed for IP at a dilution of 0.5 mg/ml. Protein A Sepharose beads were incubated with the sample for 1 h at 4°C under rotation to preabsorb any protein that nonspecifically binds to the protein A Sepharose beads. The supernatant was recovered by centrifugation and 3 μg of anti-A_{2A}R antibody (Millipore) or irrelevant IgG (for negative control) was added and incubated for 3 h at 4°C under rotation. To pull down the immune complexes, samples were then incubated with protein A Sepharose beads for 2 h at 4°C and centrifuged. The pellets were washed twice in IPB with 1% Triton X-100, 3 times in IPB with 1% Triton X-100 and 500 mM NaCl, and twice in IPB. The input (5% of the initial sample), 20% of the supernatant of both pulldowns, as well as 100% of the immunoprecipitates were resolved in RIPA buffer, and Western blots were performed with anti-A_{2A}R or anti-CD73 antibodies (see Western blot).

Western blot. Western blotting was performed as previously described (Rebola et al., 2005), using nonreducing conditions for rabbit anti-murine CD73. Incubation with the primary antibodies, namely rabbit anti-GFAP (1:20,000, Dakocytomation), mouse anti-synaptophysin (1:50,000, Sigma), mouse anti-syntaxin (1:50,000, Sigma), mouse anti-PSD-95 (1:100,000, Sigma), mouse anti-β-actin (1:20,000, Sigma), mouse anti-A_{2A}R (1:1000, Millipore), and rabbit anti-murine CD73 (1:1000, Fausther et al., 2012), all diluted in Tris-buffered saline (137 mM NaCl and 20 mM Tris-HCl, pH 7.6) with 0.1% Tween (TBS-T) and 5% BSA (fatty acid free), was performed overnight at 4°C. After washing

twice with TBS-T, the membranes were incubated with appropriate IgG secondary antibodies conjugated with alkaline phosphatase (GE Healthcare) for 2 h at room temperature. After washing, the membranes were revealed using an ECF kit (GE Healthcare) and visualized with an imaging system (VersaDoc 3000, Bio-Rad), and the densitometric analysis of protein bands was performed using the Quantity One software (Bio-Rad).

Binding assay. The binding assays were performed as previously described (Matos et al., 2012a). Briefly, the total membranes (see total membranes preparation) were resuspended in a preincubation solution (containing 50 mM Tris, 1 mM EDTA, 2 mM EGTA, pH 7.4) and a sample was collected to determine the protein concentration using the BCA assay (Thermo Scientific). Adenosine deaminase (ADA; 2 U/ml, Roche) was added and the membranes were incubated for 30 min at 37°C to remove endogenous adenosine. The mixtures were centrifuged at 25,000 × *g* for 20 min at 4°C, and the pelleted membranes were resuspended in Tris-Mg solution (containing 50 mM Tris and 10 mM $MgCl_2$, for A_{2A}R binding, or 50 mM Tris and 2 mM $MgCl_2$, for A_1R binding, pH 7.4) with 4 U/ml ADA. Binding with the selective A_{2A}R antagonist [^{3}H] 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino] ethyl)phenol (ZM241385; 3 nM; PerkinElmer) was performed for 1 h, and binding with the selective A_1R antagonist [^{3}H]1,3-dipropyl-8-cyclopenthylxanthine (DPCPX; 2 nM; PerkinElmer) was performed for 2 h, both at room temperature with 0.1–0.2 mg of protein, with constant swirling. The binding reactions were stopped by the addition of 4 ml of ice-cold Tris-Mg solution and filtration through Whatman GF/C glass microfiber filters (GE Healthcare) in a filtration system (Millipore). The radioactivity was measured after adding 5 ml of scintillation liquid (PerkinElmer). The specific binding was expressed in femtomoles per milligram of protein and was estimated by subtraction of the nonspecific binding, which was measured in the presence of 12 μM xanthine amine congener (Sigma), a mixed A_1R/A_{2A}R antagonist. All binding assays were performed in duplicate.

cAMP measurement. Striatal synaptosomes were prepared as previously described (Rebola et al., 2003). Briefly, the synaptosomal fraction was resuspended in KHR with ADA (4 U/ml; Roche) and DPCPX (50 nM; Tocris Bioscience) and was incubated for 10 min at 37°C, to eliminate endogenous adenosine and eliminate putative A_1R-mediated effects. The mixture was then incubated with (*2R,3R,4S,5R*)-2-(6-amino-2-(2-cyclohexylethylthio)-9H-purin-9-yl)-5-(hydroxymethyl) tetrahydrofuran-3,4-diol (PSB-12404; 50 nM) or (*2R,3S,4R,5R*)-5-(6-amino-2-(2-cyclohexylethylthio)-9H-purin-9-yl)-3,4-dihydroxy-tetrahydro-furan-2-yl)methylphosphoric acid triethylammonium salt (PSB-12405; 50 nM) for 10 min at 37°C, and the cAMP levels were measured as previously described (Chen et al., 2010). Briefly, the reaction was terminated by the addition of 5% ice-cold trichloroacetic acid (Ricca Chemical Company) and centrifuged for 10 min at 600 × *g* to pellet the debris after homogenization. The trichloroacetic acid was extracted from the supernatant with water-saturated ether (Alfa Aesar). The aqueous extract was dried overnight and reconstituted in assay buffer. The samples were acetylated, and the levels of cAMP accumulated in synaptosomes were determined using a cAMP Complete ELISA kit (Assay Designs) according to the manufacturer's instructions.

Inorganic free phosphate measurement. The activity of ecto-5′-nucleotidase was evaluated by measuring the formation of inorganic phosphate upon addition of AMP to striatal synaptosomes (Chan et al., 1986). Briefly, the synaptosomal fraction, prepared as previously described (Rebola et al., 2003), was resuspended in KHR and incubated for 5 min at 37°C to stabilize, followed by incubation with the tissue-nonspecific alkaline phosphatase inhibitor (TNAP-I; 10 μM; Calbiochem) and/or AMP (1 mM; Acros Organics) for 30 min at 37°C. The mixture was then centrifuged at 14 000 × *g* at 4°C for 12 min, and the inorganic free phosphate levels were measured from the supernatant using a Malachite Green Phosphate Assay kit (Cayman Chemical) according to the manufacturer's instructions.

Immunohistochemistry. Mice were anesthetized with avertin, and brain fixation was performed through transcardiac perfusion with 4% paraformaldehyde in PBS, postfixation overnight in PBS with 4% paraformaldehyde, and cryopreservation in PBS containing 25% sucrose. Frozen brains were sectioned (30 μm coronal slices) with a Leica Microsystems CM3050S cryostat. The sections were first rinsed for 5 min with PBS at room temperature, and then permeabilized and blocked with PBS containing 0.2% Triton X-100 and 5% donkey serum for 1 h, incubated in the presence of the rabbit anti-murine CD73 antibody (1:500; Fausther et al., 2012) and/or mouse anti-A_{2A}R antibody (1:500; Millipore) overnight at room temperature, rinsed three times for 10 min in PBS, and then incubated with donkey anti-mouse and/or donkey anti-rabbit secondary antibodies conjugated with a fluorophore (1:200; Alexa Fluor 488 or Alexa Fluor 555, Invitrogen) for 2 h at room temperature. After rinsing three times for 10 min in PBS, the sections were mounted on slides and allowed to dry. Vectashield mounting medium with DAPI (Vector Laboratories) was applied as well as the cover glass. All sections were examined under a fluorescence Nikon Eclipse E600 microscope, with SPOT software 4.7 (Diagnostic Instruments).

Proximity ligation assay. The PLA was performed as previously described (Trifilieff et al., 2011) in brain sections prepared as described above. The sections were first rinsed in TBS (0.1 M Tris, pH 7.4, and 0.9% w/v NaCl) at room temperature, and then permeabilized and blocked with TBS with 1% BSA and 0.5% Triton X-100 for 2 h at room temperature. The slices were incubated with the primary antibodies, namely rabbit anti-murine CD73 (1:300; Fausther et al., 2012) and anti-A_{2A}R (1:300; Millipore) overnight at room temperature. After washing four times (30 min each) in TBS with 0.2% Triton X-100, the slices were incubated for 2 h at 37°C with the PLA secondary probes (1:5; Olink Bioscience) diluted under gentle agitation. After washing twice for 5 min with Duolink II Wash Buffer A (Olink Bioscience) with agitation at room temperature, the slices were incubated with the ligation-ligase solution (Olink Bioscience) for 30 min at 37°C. After washing twice for 2 min with Duolink II Wash Buffer A with agitation at room temperature, the slices were incubated with polymerase (1:40; Olink Bioscience) in the amplification solution (Olink Bioscience) for 100 min at 37°C under gentle agitation. After washing in decreasing concentrations (2×, 1×, 0.2×, 0.02× for 10 min each) of SSC buffers (Olink Bioscience), slices were mounted on slides and allowed to dry, and coverslips were placed with Duolink Mounting Medium (Olink Bioscience). Fluorescence images were acquired on an Axiovert 200M inverted confocal microscope (Carl Zeiss Microscopy) using a 40× objective, and the PLA puncta signals were quantified with the ImageJ software, using a manual threshold to discriminate PLA puncta from background fluorescence. The built-in macro "Analyze Particles" was then used to count and characterize all objects in the thresholded image. Objects larger than 5 μm^2 were rejected, thereby effectively removing nuclei. The remaining objects were counted as PLA puncta.

Drug treatments and locomotor activity. The activity of the mice was assessed in standard polypropylene cages (15 × 25 cm) and recorded with infrared photobeams (San Diego Instrument) in 5 min bins. Before drug treatments, the mice were habituated to the new room and new cage (except in the habituation experiment) for at least 2 h. Two hours before the administration of 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo [4,3-e]-1,2,4-triazolo-[1,5-c]pyrimidine (SCH58261; 3 mg/kg, i.p.; Tocris Bioscience), the animals were challenged with vehicle (intraperitoneally, 75% saline, 15% dimethylsulfoxide, 10% castor oil). In the amphetamine (2.5 mg/kg, i.p.; Sigma) paradigm, the mice were injected in the same environment for 8 consecutive days and the locomotor activity was recorded. In the experiment with the A_{2A}R agonist drug or prodrug (see below), the mice were anesthetized with isoflurane and oxygen and were stereotaxically injected bilaterally in nucleus accumbens (anteroposterior = +1 mm from bregma; mediolateral = ±0.8 mm from midline; dorsoventral = 4.4 mm from the skull surface) with 1 μl of 2 mM solution (2 nmol) per side at a rate of 0.2 μl/min. The activity was recorded after the mice recovered from the surgery.

The A_{2A}R agonist PSB-12404 and its phosphate prodrug PSB-12405 were synthesized as previously described (El-Tayeb et al., 2009).

Working memory. We first assessed working memory in a spontaneous alternation paradigm assessed in a Y-maze. Individual mice were placed at the end of one arm and allowed to freely explore the maze for 5 min. The sequence of entrance in each arm was recorded, and the number of

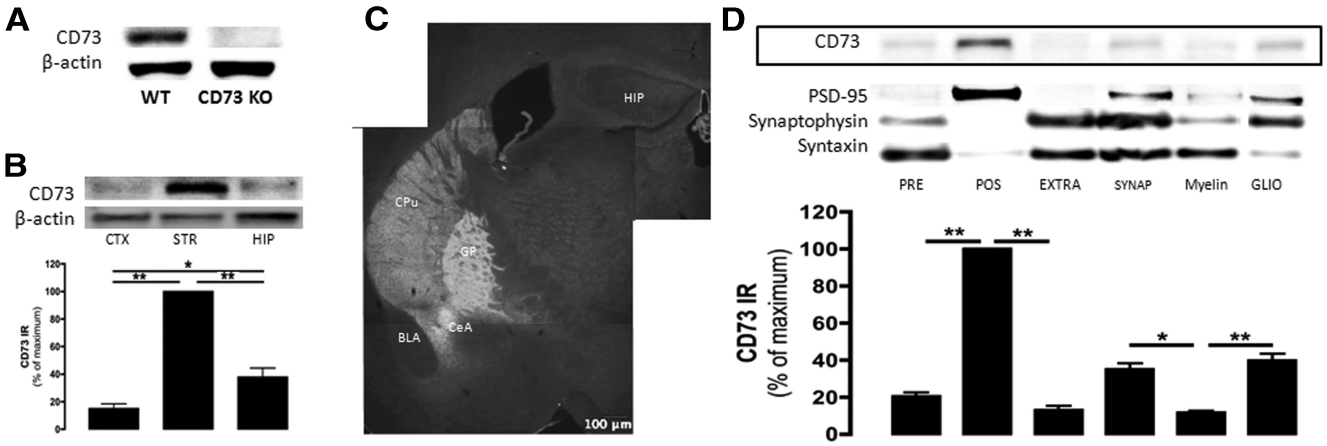

Figure 1. CD73 has a brain and subcellular distribution similar to that of A_{2A}Rs. ***A***, ***B***, Using an antibody selectively recognizing CD73 in the striatum of WT but not of CD73 KO mice, as shown in ***A*** (representative of $n = 4$), a Western blot analysis (***B***) revealed that CD73 immunoreactivity was more densely located in the striatum (STR) than in the hippocampus (HIP) or cortex (CTX) ($n = 3$). ***C***, Immunohistochemistry showed that CD73 displayed greater abundance in the globus pallidus (GP) and central nucleus of amygdala (CeA), than in the caudate-putamen (CPu), than in the hippocampus (HIP) and the basolateral amygdala (BLA) (image representative of $n = 5$). ***D***, In the striatum, subcellular fractionation ($n = 2$) showed that CD73 had a higher density in postsynaptic density (POS), when compared with presynaptic (PRE) and extrasynaptic (EXTRA) fractions and was more densely located in synaptosomes (SYNAP) and gliosomes (GLIO) than in myelin preparations. Data are the mean ± SEM, and a one-way ANOVA test was used followed by a Tukey's multiple-comparison test. $^{*}p < 0.01$; $^{**}p < 0.001$.

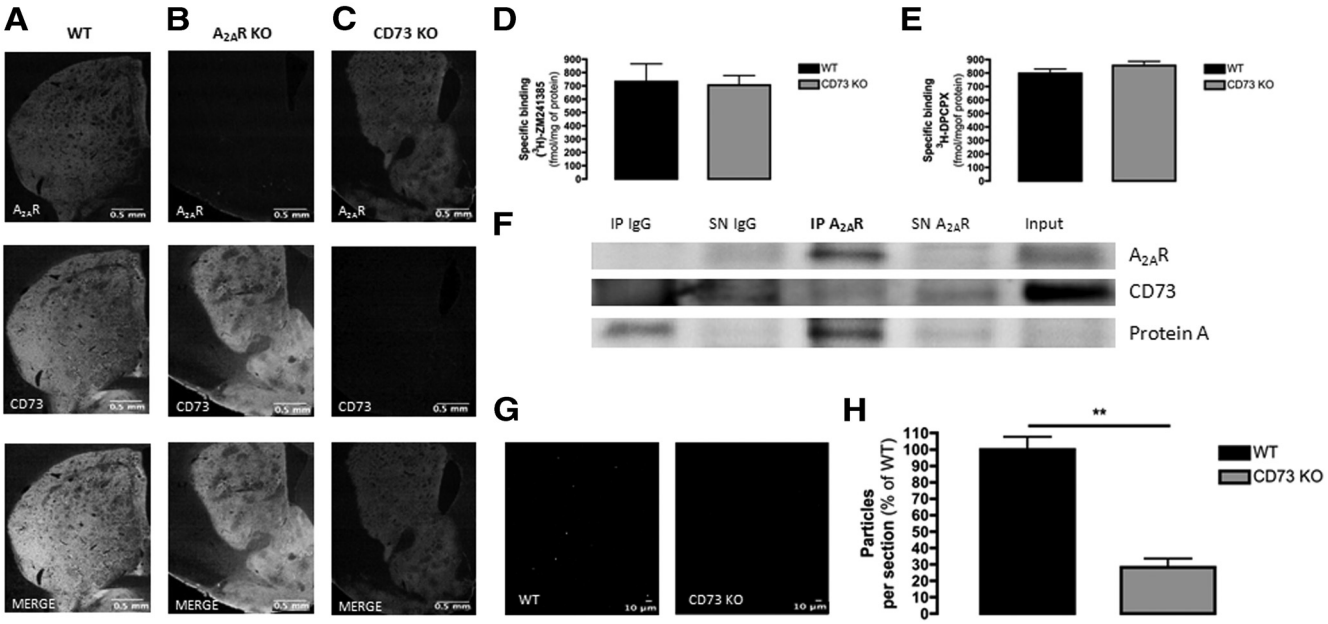

Figure 2. Colocalization and physical association of CD73 and A_{2A}Rs in the striatum. ***A–C***, Macroscopic immunohistochemical colocalization of CD73 and A_{2A}Rs in striatal slices (***A***; $n = 5$), with no immunoreactivity for CD73 in CD73 KO mice (***B***; $n = 5$), and no immunoreactivity for A_{2A}Rs in A_{2A}R KO mice (***C***; $n = 5$). ***D***, ***E***, CD73 KO mice had an identical density of binding sites for A_{2A}Rs (***D***) and A_1Rs (***E***) when compared with their WT littermates ($n = 5–6$). ***F***, The pulldown of A_{2A}Rs from striatal tissue revealed the co-immunoprecipitation of CD73; indeed, the pulldown of A_{2A}Rs (IP A_{2A}R) revealed the presence of A_{2A}Rs (top strip of blots), CD73 (middle strip of blots), and protein A (bottom strip of blots), which were also present in the pulldown of IgG (IP IgG); however, this condition (IP IgG) does not show immunoreactivity for A_{2A}Rs (top strip of blots) or CD73 (middle strip of blots). The input represents 5% of the sample before the pulldown, SN A_{2A}R and SN IgG represent 20% of the supernatant of A_{2A}R and IgG pulldown, respectively (results representative of $n = 3$). ***G***, ***H***, The intimate proximity between A_{2A}Rs and CD73 was further shown by the signal recorded in a PLA in sections of the striatum from WT mice but not from CD73 KO mice. ***G*** shows representative PLA images of striatal sections from WT mice and CD73 KO mice (representative from $n = 3$) and ***H*** displays the quantification of the PLA experiments, with the WT having 100.0 ± 7.758 positive signals per slice and the CD73 KO having significantly less PLA signal (28.27 ± 5.317). The data are the mean ± SEM. $^{**}p < 0.001$ using a Student's *t* test.

alternations (sequential entrance in the three different arms) was quantified. The percentage of spontaneous alternation consists in the percentage of alternations of the total possible number of alternations (total number of arm changes − 2).

We also assessed working memory in a more sensitive test using a radial arm maze (RAM) with eight arms as previously described (Singer et al., 2012). To motivate performance in the RAM memory tasks, the animals were maintained on a food deprivation regime, which was gradually introduced with a progressive reduction of the daily available food, until the animals reached a stable weight of not less than 85% of their *ad libitum* weight, at which time the food provided was stabilized. The RAM had eight identical and equally spaced arms (56 cm long, 12 cm wide) radiating from a central octagonal platform (side length = 12 cm). The mice were exposed to the maze for 5 min each day with a food reward at the end of each arm. The habituation was performed until the animals finished the task within 5 min. In the four baited arms paradigm, four of the eight arms were randomly set with a food reward and the mice were allowed to freely explore the maze until they ate the four food rewards. In

the eight baited arms paradigm, the eight arms were set with a food reward and the animals were allowed to freely explore the maze until they ate the eight food rewards. Each time a mouse re-entered an arm where the reward had already been eaten, a working memory error (WME) was scored. Different groups of animals were used in the two RAM experiments. When testing the impact of the $A_{2A}R$ and A_1R antagonists SCH58261 and DPCPX (Tocris Bioscience), the drug or its vehicle solution was intraperitoneally administered to the animals 30 min before they were placed in the maze.

Statistical analysis. Results are presented as the mean ± SEM. Data with one condition and one variable (e.g., genotype) were analyzed with Student's *t* test. Data from more than one condition (e.g., different brain's preparations) were analyzed with repeated-measures ANOVA or one-way ANOVA followed by a Tukey's multiple-comparison *post hoc* test or by a Dunnett's multiple-comparison *post hoc* test (for comparison with specific controls). Data with more than one variable (e.g., genotype and time) and condition were analyzed with a two-way ANOVA followed by Bonferroni *post hoc* tests. Unless otherwise indicated, the significance level was 95%.

Results

CD73, like $A_{2A}R$, is most abundant in the striatum with a predominant postsynaptic localization

To determine the brain distribution of CD73, we first certified the selectivity of our anti-CD73 antibody, through Western blot analysis, which we found to recognize a band (≈65 kDa) in striatal membranes of WT mice, without detectable signal in CD73 KO mice (Fig. 1*A*). We then compared the density of CD73 in different brain regions; Western blot analysis of total membranes showed that CD73 is more abundant in the striatum ($p < 0.01$) than in the hippocampus or prefrontal cortex (Fig. 1*B*). This was confirmed by immunohistochemical analysis (Fig. 1*C*), showing a higher CD73 immunoreactivity in different basal ganglia areas, as well as in the central nucleus of amygdala, when compared with the hippocampus or cerebral cortex. Within the basal ganglia, CD73 immunoreactivity was higher in the globus pallidus than in the caudate putamen and nucleus accumbens.

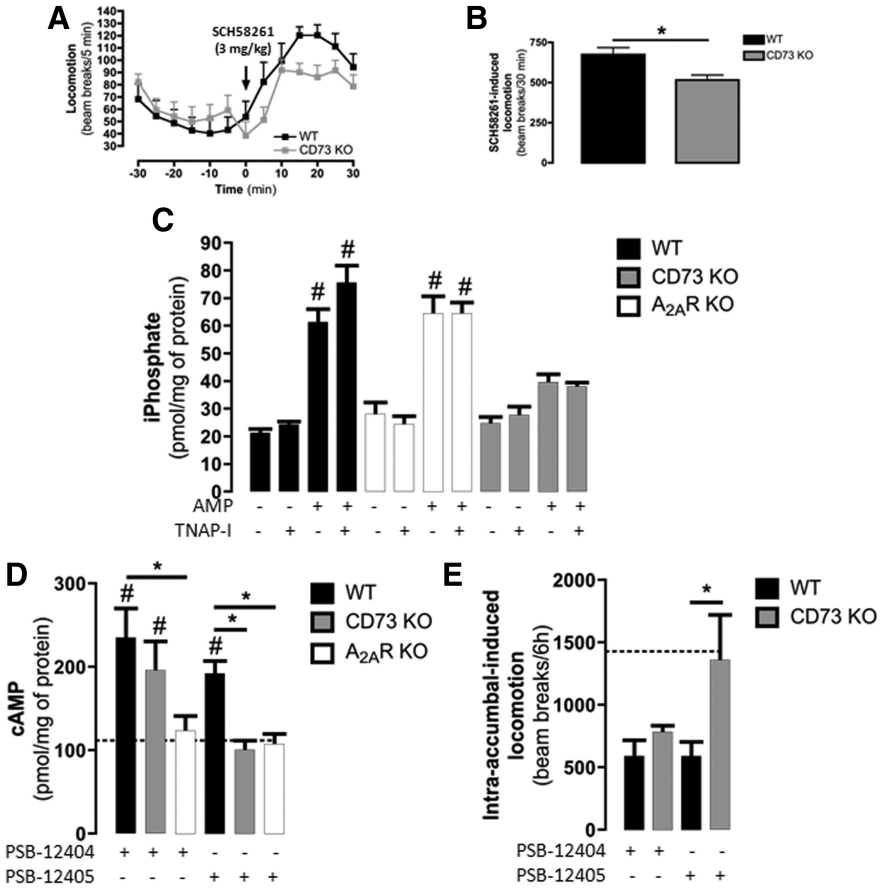

Figure 3. CD73 activity provides the adenosine activating striatal $A_{2A}Rs$. ***A***, ***B***, Acute administration of the $A_{2A}R$ antagonist SCH58261 (3 mg/kg, i.p.) induced hyperlocomotion. ***A*** represents the mean ± SEM of beam breaks per 5 min, whereas ***B*** represents the mean ± SEM of the total number of beam breaks in 30 min after SCH58261 administration in WT and CD73 KO mice ($n = 10–11$). ***C*** shows that striatal synaptosomes from both WT and $A_{2A}R$ KO mice can dephosphorylate AMP (1 mM), whereas the striatal synaptosomes from CD73 KO mice cannot ($n = 4–7$); furthermore, TNAP-I (10 μM) failed to affect the extracellular catabolism of AMP. ***D*** shows that both PSB-12405 (50 nM; a prodrug that requires CD73 activity to become an $A_{2A}R$ agonist) and PSB-12404 (50 nM; an $A_{2A}R$ agonist) enhanced cAMP levels compared with control (111.7 ± 8.275 pmol/mg protein, $n = 5$; represented by the horizontal dash line) in striatal synaptosomes from WT mice, whereas only the drug but not the prodrug enhanced cAMP in striatal synaptosomes from CD73 KO mice and neither PSB-12404 nor PSB-12405 were effective in $A_{2A}R$ KO mice ($n = 4–6$). ***E*** shows that the bilateral intra-accumbal administration of the prodrug (PSB-12405, 2 nmol) induced hypolocomotion in WT mice when compared with CD73 KO mice, whereas the drug (PSB-12404, 2 nmol) induced hypolocomotion in both groups ($n = 5–6$). The horizontal dashed line indicates the spontaneous locomotion in control mice (1427.9 ± 107.1 bean breaks/6 h, $n = 17$). The data are the mean ± SEM. $^*p < 0.05$ between genotypes (as indicated by the top bars) using a Student's *t* test in ***B*** and ***E*** or using a one-way ANOVA test followed by a Dunnett's multiple-comparison test to WT mice as control in ***D***; $^\#$differences from control (i.e., no added drugs) with $p < 0.001$ using a one-way ANOVA test followed by a Tukey's multiple-comparison test in ***C***; and $p < 0.05$ using a one-way ANOVA test followed by a Dunnett's multiple-comparison test to control in ***D***.

We next attempted to define the cellular and subsynaptic localization of CD73 in the basal ganglia. In accordance with the previously described localization of CD73 in astrocytes and neurons (Kreutzberg et al., 1978; Schoen and Kreutzberg, 1997), we found that CD73 was more abundant in gliosomes (astrocytic plasmalemmal vesicles) and synaptosomes than in myelin membranes (Fig. 1*D*). Furthermore, within synapses CD73 was more abundantly located in the postsynaptic density than in the presynaptic active zone and was scarcely located in perisynaptic regions (extrasynaptic fraction) (Fig. 1*D*). Altogether, this striatum-enriched pattern of CD73 together with its predominant postsynaptic localization suggests that CD73 is concentrated in the dendrites of striatal medium spiny neurons, precisely where $A_{2A}Rs$ are more densely present, a conclusion that awaits to be confirmed by electronic microscopy studies.

Colocalization and physical association of CD73 and $A_{2A}Rs$ in the striatum

The observed similar greater abundance of CD73 and $A_{2A}Rs$ in the striatum together with the proposed functional association between ATP-derived adenosine and the activation of $A_{2A}R$ (Cunha et al., 1996; Rebola et al., 2008), led us to investigate the association between CD73 and $A_{2A}R$. Double immunohistochemistry analysis showed that CD73 colocalized with $A_{2A}R$ in the striatum (Fig. 2*A*). We confirmed the selectivity of each antibody labeling by showing the absence of the putative $A_{2A}R$ signal in $A_{2A}R$ KO mice (Fig. 2*B*) and the absence of the putative CD73

signal in CD73 KO mice (Fig. 2*C*); furthermore, there do not seem be overt changes of CD73 immunoreactivity in A_{2A}R KO mice (Fig. 2*B*) or of A_{2A}R signal in CD73 KO mice (Fig. 2*C*). In agreement, no changes were found in the binding density of either the selective A_{2A}R antagonist ([^{3}H]ZM 241385; Fig. 2*D*) or of the selective A_1R antagonist ([^{3}H]DPCPX; Fig. 2*E*) to total striatal membranes of CD73 KO mice compared with WT mice.

The physical interaction between A_{2A}Rs and CD73 was further prompted by the observation that the pulldown of striatal A_{2A}R revealed a co-immunoprecipitation with CD73 (Fig. 2*F*). To consolidate this suggested association between CD73 and A_{2A}Rs in the striatum, we used a PLA approach that showed a selective physical proximity (≤16 nm) between A_{2A}R and CD73 in WT but not CD73 KO mice (Fig. 2*G*,*H*).

CD73-derived adenosine is required for striatal A_{2A}R activation and function

Since it is well documented that A_{2A}R antagonists trigger a hyperlocomotion through the blockade of striatopallidal A_{2A}Rs (Shen et al., 2008), we tested whether CD73 would be responsible for the formation of the adenosine tonically activating this population of A_{2A}Rs. In accordance with this hypothesis, we report that the hyperlocomotion triggered by the selective A_{2A}R antagonist SCH58261 (3 mg/kg, i.p.) had significantly ($p < 0.01$) lower amplitude in CD73 KO mice when compared with their WT littermates (Fig. 3*A*,*B*). This is probably due to the lack of AMP-derived adenosine to tonically activate striatal A_{2A}Rs since we now report that striatal synaptosomes from WT and A_{2A}R KO mice were able to dephosphorylate AMP, as gauged from the formation of inorganic phosphate, whereas this did not occur in striatal synaptosomes from CD73 KO mice (Fig. 3*C*). This conclusion reached in other brain preparations that CD73 is the predominant activity responsible for the formation of adenosine from extracellular AMP, as further confirmed by the lack of impact of the alkaline phosphatase inhibitor TNAP-I (10 μM) on the extracellular catabolism of AMP in striatal synaptosomes from either WT or CD73 KO mice (Fig. 3*C*) extends to striatal preparations.

To reinforce the direct relation between CD73 and A_{2A}R in the striatum, we took advantage of a novel A_{2A}R pro-agonist (PSB-12405), which needs to be dephosphorylated by CD73 to generate the active form of the A_{2A}R agonist PSB-12404 (El-Tayeb et al., 2009; Flögel et al., 2012). We first confirmed in striatal synaptosomes that the pro-agonist indeed activated A_{2A}Rs in a CD73-dependent manner, by comparing the ability of the drug and prodrug to enhance cAMP levels, an established measure of A_{2A}R activity in the striatum (Svenningsson et al., 1998; Corvol et al., 2001). We found that the prodrug increased cAMP levels in striatal synaptosomes from WT mice, but not in those from either CD73 KO or A_{2A}R KO mice (Fig. 3*D*); whereas, the A_{2A}R agonist (PSB-12404) increased cAMP levels in WT and CD73 KO mice, but not in A_{2A}R KO mice (Fig. 3*D*). This shows that PSB-12405 requires CD73 activity to activate striatal A_{2A}Rs, which allows using this pro-agonist to test whether CD73 is responsible for generating the adenosine that specifically controls the impact of A_{2A}Rs on striatal-related behavioral responses.

In agreement with the previously reported hypolocomotor effect of A_{2A}R agonists directly injected in the nucleus accumbens (Hauber and Münkle, 1997; Nagel et al., 2003), the bilateral intra-accumbal injection of PSB-12405 reduced locomotion in the WT mice to an extent greater than that in the CD73 KO mice (Fig. 3*E*). Instead, when PSB-12404 was injected no differences were found between the two genotypes (Fig. 3*E*).

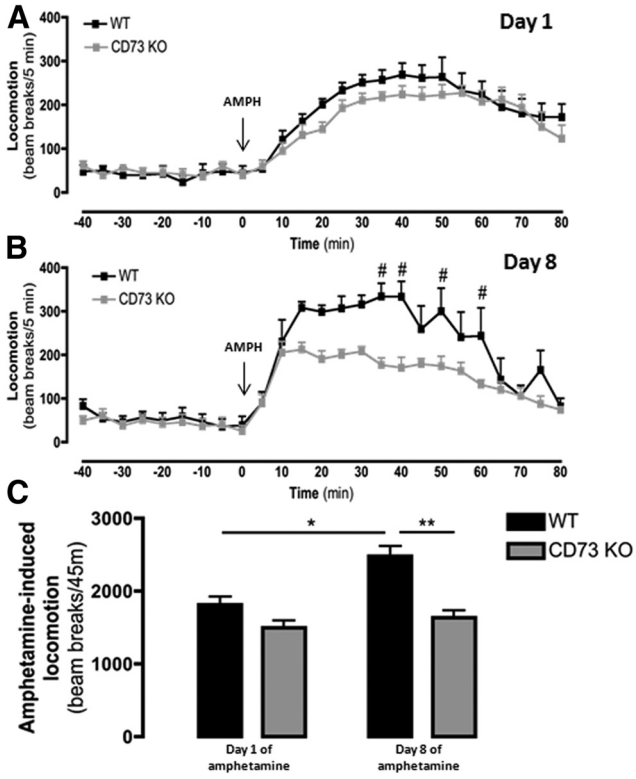

Figure 4. CD73 KO mice show no locomotor sensitization to amphetamine. CD73 KO ($n = 7$) and WT ($n = 6$) mice showed identical hyperlocomotion induced by an acute single administration of amphetamine (***A***). However, CD73 KO mice showed no sensitization to amphetamine after being challenged for 8 consecutive days, in contrast to the WT mice that showed a significant increase in locomotion (***B***, ***C***). Data are the mean ± SEM. $^{\#}p < 0.05$, using a two-way ANOVA followed by Bonferroni *post hoc* tests in ***B***; $^{*}p < 0.01$, using repeated-measures ANOVA; $^{**}p < 0.001$, using a two-way ANOVA followed by Bonferroni *post hoc* test in ***C***.

CD73 KO mice show no sensitization to amphetamine, mimicking A_{2A}R KO phenotype

It was previously shown that global-A_{2A}R KO mice (Chen et al., 2003), as well as forebrain-A_{2A}R KO mice (Bastia et al., 2005), do not develop psychomotor sensitization to amphetamine, which provides an additional opportunity to probe the functional association between CD73 and the activation of A_{2A}Rs.

CD73 depletion had no effect on the locomotor response to habituation to a novel environment or to the habituation to a saline injection (data not shown). Furthermore, there was no difference between CD73 KO and WT mice in the locomotor response to the first administration of a low dose (2.5 mg/kg) of amphetamine (Fig. 4*A*,*C*). However, continued daily treatment with this low dose of amphetamine markedly enhanced (sensitized) locomotor responses in control WT mice ($p < 0.05$, day 8 vs day 1), whereas no sensitization to amphetamine was observed in CD73 KO mice (Fig. 4*B*,*C*).

CD73 KO mice display improved working memory, mimicking the phenotype by genetic and pharmacological inactivation of A_{2A}Rs

It was previously shown that A_{2A}Rs control working memory performance: indeed, it is deficient in A_{2A}R-overexpressing mice (Giménez-Llort et al., 2007) and improved in global-A_{2A}R KO mice as well as forebrain-A_{2A}R KO and striatal-A_{2A}R KO mice (Zhou et al., 2009; Wei et al., 2011). We now report that CD73 KO mice displayed an improved working memory compared

with WT mice, when tested in the spontaneous alternation paradigm in a Y-maze (Fig. 5*A*), without changes in their locomotion (Fig. 5*B*). In addition, CD73 KO mice made fewer working memory errors than WT mice in the eight baited arms version of the eight radial arm maze (Fig. 5*C*,*D*). This result was further validated in the four baited arms version of the eight radial arm maze with a separate group of mice (data not shown). Thus, despite the limitations of each test in the evaluation of working memory, taken together, these results show a consistent improvement of working memory performance when CD73 is depleted. To discount developmental changes in the KO lines, we tested the effect of an acute blockade of A_{2A}Rs in two different paradigms of working memory, using a selective A_{2A}R antagonist (SCH58261, 0.03 mg/kg, i.p.; the same dose that was able to blunt amphetamine sensitization without changing basal locomotion; Bastia et al., 2005). To discard the involvement of the other main adenosine receptor in the phenotype, we also tested the effect of an acute blockade of A_1Rs using a selective A_1R antagonist (DPCPX, 0.03 mg/kg, i.p.) in the same paradigms. The mice that received SCH58261 30 min before testing displayed an improved working memory compared with the mice that received either vehicle (control) or DPCPX, when tested in the spontaneous alternation paradigm in the Y-maze test (Fig. 5*E*), without changes in their locomotion (Fig. 5*F*). A similar result was obtained with a higher dose of DPCPX (0.3 mg/kg, i.p.; data not shown). In addition, mice treated with SCH58261 made fewer working memory errors than either control or DPCPX-treated mice in the eight baited arms (Fig. 5*G*,*H*) version of the eight radial arm maze. This phenotype resulting from the pharmacological blockade of A_{2A}Rs and not A_1Rs is superimposable to that of A_{2A}R KO mice and also parallels that of CD73 KO mice, further strengthening our contention that CD73 is responsible for the formation of the adenosine that tonically activates striatal A_{2A}Rs.

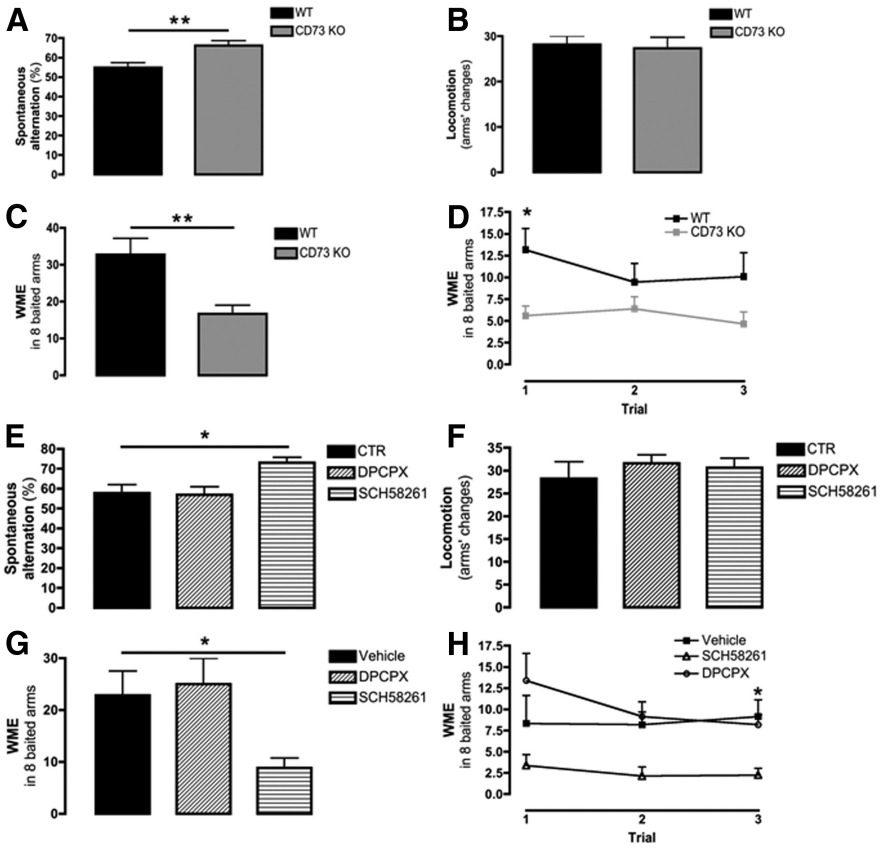

Figure 5. CD73 KO mice display an improved working memory, which is mimicked by the acute blockade of A_{2A}Rs, but not A_1Rs. CD73 KO mice have an improved working memory when tested in a Y-maze paradigm (***A***) analyzing their spontaneous alternation in comparison with the WT littermates, with no differences in locomotion (***B***), evaluated by the number of arm changes (n = 11–12). In the radial arm maze with eight baited arms, CD73 KO displayed significantly fewer WMEs (scored each time an animal re-enter in a previous visited arm) in comparison with the WT littermates (n = 11–12) (***C***, ***D***). Mice acutely treated with a selective A_{2A}R antagonist (SCH58261, 0.03 mg/kg, i.p.) had an improved working memory when tested in a Y-maze paradigm, analyzing their spontaneous alternation in comparison with the control group (vehicle, intraperitoneally), with no differences in the mice treated with a selective A_1R antagonist (DPCPX, 0.03 mg/kg, i.p.) (***E***), and no differences in locomotion (***F***), evaluated by the number of arm changes (n = 8). In the radial arm maze with eight baited arms, SCH58261-treated mice displayed significantly fewer WMEs in comparison with the control group, with no differences in the mice treated with a selective A_1R antagonist (n = 8). Data are the mean ± SEM. *$p < 0.05$ and **$p < 0.01$ using a Student's t test (***A–C***), a one-way ANOVA test followed by a Dunnett's multiple-comparison test to control (***E–G***), or a two-way ANOVA followed by Bonferroni *post hoc* test (***D***, ***H***).

Discussion

We here showed that the activity of CD73, the major enzyme dephosphorylating AMP to adenosine in the CNS, and therefore responsible for the last enzymatic step in the formation of extracellular ATP-derived adenosine, has a crucial role in the activation of striatal A_{2A}Rs. The intrinsic relation between CD73 and A_{2A}Rs is supported by their anatomical localization and physical proximity in the striatum. The colocalization of CD73 and A_{2A}Rs in the striatum is demonstrated by their similar distribution patterns in the basal ganglia as well as by the postsynaptic enrichment of these two molecules and their physical proximity, documented by co-immunoprecipitation and proximity ligation assays. After showing that the deletion of CD73 abolished the extracellular dephosphorylation of AMP, the functional association between CD73 activity and the activation of striatal A_{2A}Rs was validated by the abolishment of *ex vivo* (i.e., cAMP formation) as well as *in vivo* effects (hypolocomotor) of a novel prodrug for A_{2A}R agonism (El-Tayeb et al., 2009; Flögel et al., 2012) in either CD73 KO or A_{2A}R KO mice.

The functional association between CD73 activity and the activation of striatal A_{2A}Rs was further confirmed *in vivo*, in three major behavioral responses that have previously been shown to involve A_{2A}R activation (i.e., hypolocomotion, decreased working memory and behavioral sensitization to psychoactive drugs). Thus, we here showed that CD73 KO mice display a reduced hyperlocomotor response to a supramaximal dose of a selective A_{2A}R antagonist (SCH58261), which indicates that CD73 KO mice have less adenosine that is selectively activating striatal A_{2A}Rs responsible for the hyperlocomotor effect (Yu et al., 2008). We are not suggesting that CD73 KO mice have in general lower levels of adenosine since they display a normal A_1R-mediated control of synaptic transmission (Zhang et al., 2012); instead, these data indicate that CD73 KO mice have lower levels of adenosine near CD73–A_{2A}R complexes. In addition, we have previously shown that the inactivation of A_{2A}Rs (Chen et al., 2003),

namely of forebrain A_{2A}Rs, as well as acute A_{2A}R blockade by SCH58261 (0.03 mg/kg) (Bastia et al., 2005), abolishes the psychomotor sensitization to amphetamine, without changing the basal locomotion; notably, we now report a similar phenotype in CD73 KO mice. It was also shown that inactivation of A_{2A}Rs (Zhou et al., 2009), namely striatal A_{2A}Rs (Wei et al., 2011), enhances working memory performance, a similar phenotype as now observed after A_{2A}R antagonist administration, as well as in CD73 KO mice, but not after A_1R antagonist administration. All together, the parallel modifications of these behavioral responses by eliminating CD73 or A_{2A}Rs but not A_1Rs (Giménez-Llort et al., 2002, 2005), as well as upon acute A_{2A}Rs, but not A_1R blockade, prompt the conclusion that CD73 is responsible for the formation of the adenosine that activates A_{2A}Rs in the striatum.

This proposed activation of A_{2A}Rs selectively by CD73-mediated formation of adenosine seems to be a general feature of A_{2A}Rs not only in the striatum, but also in other tissues and cell types. Indeed, it was shown that the inhibition of CD73 selectively blunts the ability of A_{2A}Rs to control synaptic plasticity in hippocampal synapses (Rebola et al., 2008) or synaptic adaptation at the neuromuscular junction (Correia-de-Sá et al., 1996; Cunha et al., 1996a), as well as the control of glutamate-induced toxicity in cultured granular cells (Boeck et al., 2007). Furthermore, the control by A_{2A}Rs of the vascular tone (Koszalka et al., 2004; Zernecke et al., 2006) and of the immune–inflammatory system has also been shown to strictly depend on the activity of CD73 (Deaglio et al., 2007; Peng et al., 2008; Flögel et al., 2012). The tight association between CD73 and A_{2A}Rs is further heralded by the observation that several conditions trigger a coordinated induction or repression of CD73 and A_{2A}R expression (Napieralski et al., 2003; Deaglio et al., 2007), strongly supporting the view that these two molecules are tightly interconnected.

Notably, there seems to be a selective association of CD73-mediated formation of adenosine with the activation of facilitatory A_{2A}Rs rather than with the more abundant inhibitory A_1Rs in the nervous system (for review, see Fredholm et al., 2005). Indeed, several groups concluded that the inhibition or genetic deletion of CD73 failed to affect the modulation of synaptic transmission by A_1Rs either in physiological or pathological conditions (Lloyd et al., 1993; Brundege and Dunwiddie, 1996; Cunha et al., 1996; Lovatt et al., 2012; Zhang et al., 2012), in contrast to the conclusions derived from a transgenic mouse with hampered release of gliotransmitters (Pascual et al., 2005). This dissociation between CD73 activity and A_1R activation is further supported by the different localization of CD73 and A_1Rs throughout the brain (Lee et al., 1986; Fastbom et al., 1987). This is in general agreement with the idea that the activation of A_1Rs results from the activity-dependent metabolic control of adenosine kinase (Diógenes et al., 2012) producing a direct outflow of adenosine as such (Lloyd et al., 1993; Brundege and Dunwiddie, 1998; Frenguelli et al., 2003). However, it cannot be excluded that ATP-derived adenosine might also activate A_1Rs in particular systems, such as in the control of tubuloglomerular feedback (Thomson et al., 2000) or of nociception, which requires the participation of alkaline phosphatase (Zylka et al., 2008; Sowa et al., 2010), which we now ruled out to contribute for the extracellular catabolism of AMP in striatal synapses.

This selective activation of A_{2A}Rs by CD73-mediated adenosine formation provides direct support to the previous proposal to understand the differential activation of inhibitory A_1Rs and facilitatory A_{2A}Rs according to the functional needs of neuronal circuits (Cunha, 2008). Thus, it is proposed that the activation of synaptic A_{2A}Rs (Rebola et al., 2005) is designed for local adaptive functional changes that are driven by activity-dependent experience (Cunha, 2008); therefore, the source of the adenosine designed to activate A_{2A}Rs should be locally produced, solely within the recruited synapses. The presently observed localization of CD73 within synapses (see also Cunha et al., 2000), mainly at the postsynaptic density, contributes to this main aim of converting the activity-dependent ATP release from synapses (Wieraszko et al., 1989; Cunha et al., 1996b; Pankratov et al., 2006) into the adenosine responsible for the local activation of A_{2A}Rs. In addition to astrocytic release of ATP (Halassa et al., 2009; Schmitt et al., 2012), the localization of the newly identified vesicular nucleotide transport within synapses, namely at the nerve terminal (Larsson et al., 2012), heralds our proposal of a local synaptic release of ATP as the possible source of neuronal CD73-mediated adenosine signaling acting through A_{2A}Rs. The present study focused only on the relation between adenosine formation and A_{2A}R activation; it remains to be explored whether the clearance of adenosine by the large family of nucleoside transporters (Parkinson et al., 2011), which activity is controlled by A_{2A}Rs in synapses (Duarte-Pinto et al., 2005), might also play a role in restraining CD73-generated adenosine for the activation of A_{2A}Rs, as was recently proposed (Nam et al., 2013).

It also remains to be defined whether the association between CD73-mediated formation of ATP-derived adenosine and the activation of A_{2A}Rs observed under near-physiological conditions can also be extrapolated to pathological brain conditions. Indeed, A_{2A}R blockade is established to afford a robust neuroprotection in animal models of brain diseases ranging from Alzheimer's or Parkinson's diseases to epilepsy or ischemia (Chen et al., 1999, 2001; El Yacoubi et al., 2008; Canas et al., 2009). However, despite the extensive characterization of the role of A_{2A}Rs, its source of adenosine has been unclear. Remarkably, noxious brain conditions trigger an enhancement of the extracellular levels of ATP (Di Virgilio, 2000). Since we confirmed that the extracellular conversion of AMP into adenosine seems to be wiped out in CD73 KO mice (Klyuch et al., 2012; Lovatt et al., 2012; Zhang et al., 2012), with no compensation of alternative enzymatic activities such as alkaline phosphatase (Langer et al., 2008), it is tempting to consider the possibility that the manipulation of CD73 might afford a benefit similar to that observed for A_{2A}R blockade (for review, see Cunha, 2005; Chen et al., 2007). This might eventually provide a functional role for the localization of CD73 in astrocytic membranes (Kreutzberg et al., 1978), now also confirmed to be present in gliosomes, which joins the proposed role of glial A_{2A}Rs in neurodegeneration (Yu et al., 2008; Matos et al., 2012b).

In summary, the present study provides the first molecular and behavioral demonstration that CD73 activity is responsible for the formation of the adenosine that activates striatal A_{2A}Rs. Therefore, our work points to CD73 as a new target that can fine tune A_{2A}R activity, paving the way to consider CD73 as a potential alternative target to A_{2A}Rs to manipulate activity-dependent synaptic adaptation and eventually neurodegeneration.

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