Purinergic Signalling (2014) 10:523–528
DOI 10.1007/s11302-014-9417-4

BRIEF COMMUNICATION

Striatal adenosine A_{2A} receptor expression is controlled by S-adenosyl-L-methionine-mediated methylation

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Received: 11 April 2014 / Accepted: 5 June 2014 / Published online: 19 June 2014
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Abstract Adenosine A_{2A} receptor ($A_{2A}R$) is a G protein-coupled receptor enriched in the striatum for which an increased expression has been demonstrated in certain neurological diseases. Interestingly, previous in vitro studies demonstrated that $A_{2A}R$ expression levels are reduced after treatment with S-adenosyl-L-methionine (SAM), a methyl donor molecule involved in the methylation of important biological structures such as DNA, proteins, and lipids. However, the in vivo effects of SAM treatment on $A_{2A}R$ expression are still obscure. Here, we demonstrated that 2 weeks of SAM treatment produced a significant reduction in the rat striatal $A_{2A}R$ messenger RNA (mRNA) and protein content as well as $A_{2A}R$-mediated signaling. Furthermore, when the content of 5-methylcytosine levels in the 5′UTR region of *ADORA2A* was analyzed, this was significantly increased in the striatum of SAM-treated animals; thus, an unambiguous correlation between SAM-mediated methylation and striatal $A_{2A}R$ expression could be established. Overall, we concluded that striatal $A_{2A}R$ functionality can be controlled by SAM treatment, an issue that might be relevant for the management of these neurological conditions that course with increased $A_{2A}R$ expression.

Keywords S-adenosyl-L-Methionine · SAM · *ADORA2A* · Adenosine A_{2A} receptor · Methylcytosine · Striatum

Introduction

Adenosine mediates its actions by the activation of specific plasma membrane G protein-coupled receptors (GPCRs) classically classified into four subtypes (A_1R, $A_{2A}R$, $A_{2B}R$, and

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A_3R) [1]. Both the A_1Rs and A_{2A}Rs are primarily responsible for the central effects of adenosine [2]. The A_{2A}R, mostly coupled to G_s/G_{olf} proteins [3], is expressed at high levels in only a few regions of the brain, namely primarily striatum, olfactory tubercle, and nucleus accumbens [4]. Interestingly, upregulation of A_{2A}R expression levels has been shown in the putamen and in peripheral blood cells from Parkinson's disease (PD) patients with levodopa-induced dyskinesias, well-correlating with the severity of the disease [5–9].

The gene that codifies human A_{2A}R (*ADORA2A*) is located at chromosome 22 [10–13], but its gene expression regulation has not been widely studied [14]. However, we previously demonstrated that DNA methylation plays a key role in both the differential *ADORA2A* content within different brain areas [15] and the receptor expression levels observed in pathological conditions such as Huntington's disease and schizophrenia [16, 17]. In addition, we also demonstrated that *ADORA2A* expression is modulated by S-adenosyl-L-methionine (SAM) treatment in cultured cells [18]. Of note, SAM participates in transmethylation, transsulfuration, and aminopropylation anabolic reactions. Accordingly, SAM constitutes the main biological methyl donor molecule involved in the methylation of DNA, proteins, and phospholipids [19–21]. Interestingly, a previous report showed that parenteral and oral SAM treatments promoted an increase in SAM levels in the cerebrospinal fluid of depressed patients, indicating that SAM crosses the blood–brain barrier [22]. Hence, considering the presented data, we previously proposed the use of SAM as an adjunctive therapy in levodopa-treated PD patients to reduce striatal A_{2A}R levels [23].

Taking into account all these considerations, here, we aimed to demonstrate that in vivo SAM treatment was indeed able to control striatal A_{2A}R expression. To this end, we monitored striatal A_{2A}R messenger RNA (mRNA) content and A_{2A}R ligand-binding avidity in both control and SAM-treated animals. In addition, the 5-methylcytosine levels in the 5′UTR region of *ADORA2A* were also analyzed, and a close relationship between SAM-mediated methylation and decline in striatal A_{2A}R expression was established.

Materials and methods

Materials

[^{3}H]ZM241385 ([2-^{3}H](4-(2-[7-amino-2-(2-fury1) [1, 2, 4] triazolo [2,3-a] [1, 3, 5] triazin-5-ylamino]ethyl)phenol 27.4 Ci/mmol) was from the American Radiolabeled Chemicals (Saint Louis, USA). SAM, theophylline, and calf intestine adenosine deaminase (ADA) were obtained from Sigma (Madrid, Spain). All other products were of analytical grade. SAM was diluted in sterile water.

Animals

Thirty rats (Sprague–Dawley, 100 g in weight) were housed with access to food and water ad libitum in a colony room kept at 19–22 °C and 40–60 % humidity under a 12:12 h light/dark cycle. All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local animal care committee of Universitat de Barcelona (99/01) and the Generalitat de Catalunya (99/1094). The rats were killed under anesthesia (400 mg/Kg chloral hydrate), and their brains were rapidly removed from the skull. The whole left striatum containing both dorsal (caudate–putamen) and ventral (nucleus accumbens) striatal areas were dissected and immediately frozen at −80 °C.

In a first set of animals (saline- and SAM-treated rats, n=5 for each group), the left striatum was removed for mRNA and DNA methylation analysis. In a second animal cohort (n=5–10 for each group), the whole left striatum was used for binding assay and cAMP determinations.

[^{3}H]ZM 241385 binding assay to striatal membranes extracts

Striatal membrane extracts from saline- and SAM-treated rats were obtained and used for A_{2A}R radioligand-binding assays as previously described [24]. Briefly, membrane extracts were incubated with 5 U/mg ADA in 50 mM Tris HCl, 2 mM $MgCl_2$, 100 mM NaCl, pH 7.4 for 30 min at 25 °C in order to eliminate endogenous adenosine from membrane preparations. Then, membrane extracts (70 μg of protein) were incubated with the specific and selective A_{2A}R antagonist [^{3}H]ZM 241385 at 40 nM for 2 h at 25 °C using 5-mM theophylline to obtain non-specific binding. Binding assays were stopped by rapid filtration through Whatman GF/B filters, which were immediately washed and counted in a Microbeta Trilux liquid scintillation counter (Wallac).

cAMP assay

Total cAMP accumulation was measured using the LANCE *Ultra* cAMP kit (PerkinElmer, Waltham, MA, USA). Striatal membrane extracts (1 μg) from saline- and SAM-treated rats were resuspended in stimulation buffer (HBSS 1X, 5 mM Hepes pH 7.4, 10 mM $MgCl_2$, 0.1 % BSA) and incubated for 20 min at room temperature. Afterwards, zardaverine (10 μM), GTP (10 μM), and ATP (150 μM) were included into the extract and were incubated for 10 min at room temperature. Subsequently, the ligands (Basal, 1 μM Forskolin and 200 nM CGS) were added for 30 min at room temperature prior to lysis. Eu-cAMP tracer and U*Light*™-anti-cAMP reagents were prepared and added to the sample according to the LANCE® *Ultra* cAMP Kit instruction

manual. Three hundred eighty-four-well plate was incubated 1 h at room temperature in the dark and was then read on a POLARstar microplate reader (BMG Labtech, Durham, NC, USA). Measurement at 620 and 665 nm were used to detect the TR-FRET signal, and the concomitant cAMP levels were calculated following the manufacturer's instructions.

RNA purification

RNA purification was carried out with RNeasy Midi kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer. The concentration of each sample was obtained from A_{260} measurements with Nanodrop 1000. RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA).

Retrotranscription reaction

The retrotranscriptase reaction (100 ng RNA/μL) was carried out by using the High-capacity cDNA Archive kit (Applied Biosystems, Madrid, Spain) following the protocol provided by the supplier.

TaqMan PCR

TaqMan PCR conditions were the same as previously described [18]. Standard curves for rat *ADORA2A*, *ADORA1*, *Drd2*, and β-glucuronidase (GUS) were prepared using serial dilutions of cDNA from wild-type rat brains. The identification numbers for rat *ADORA2A*, *ADORA1*, *Drd2*, and the endogenous control *GUS* probes were Rn00583935_m1, Rn00567668_m1, Rn01418275_m1, and Rn00566655_m1, respectively (Applied Biosystems, Madrid, Spain).

Quantitative DNA methylation analysis

DNA purification, bisulfite treatment, and quantitative DNA methylation analysis by MassArray platform of SEQUENOM were performed as described [18]. A locus located in the 5′ untranslated region (UTR) of rat *ADORA2A* gene was analyzed to learn the percentage of DNA methylation. Primers were designed using MethPrimer (http://www.urogene.org/methprimer/). The reverse primer presented a T7-promoter tagged to obtain an appropriate product for in vitro transcription and an 8-bp insert to prevent abortive cycling. The forward primer contained a 10mer-tagged to balance the PCR primer length. The sequences of primers used for amplification of bisulfite-treated DNA were (included tags are indicated below in lower case): forward, 5′-aggaagagagATTTTTTTAGTAGGAAGGAAGGGT-3′, reverse, 5′-cagtaatacgactcactatagggagaaggctAAAAAACCAAAATAACACAAACAAC -3′. The locus amplified was located at positions 16466335–16465793 of contig NC_005119.3.

Results and discussion

A fundamental goal of GPCR pharmacology is to settle receptor–ligand interactions in order to catalog receptors according to their thermodynamic and kinetic properties. Interestingly, an important aspect in drug action is not only the ligand concentration but also the receptor availability. Therefore, controlling GPCR expression constitutes a compelling way to manipulate receptor-mediated physiological responses both in normal and in pathological conditions. Hence, here, we aimed to shed light into the in vivo control of $A_{2A}R$ expression, a GPCR with eventual increased prevalence in certain neurological conditions. Indeed, $A_{2A}R$ levels have been shown to be increased in the putamen of some PD patients [5–9].

In order to evaluate whether SAM treatment was able to modify $A_{2A}R$ expression levels in rat striatum, we administered SAM (100 mg/kg, i.p.) [25] during 2 weeks, and the amount of striatal $A_{2A}R$ was monitored by means of radioligand-binding experiments using [^{3}H]ZM241385, a selective $A_{2A}R$ antagonist. Interestingly, while saline-treated animals showed a [^{3}H]ZM241385-specific binding of 571.6 ±43.7 fmol/mg protein, the SAM-treated animals exhibited a binding of 328.9±43.6 fmol/mg protein; thus, a significant reduction of ~40 % ($P<0.001$) in the $A_{2A}R$ content was achieved after SAM treatment (Fig. 1). Subsequently, we

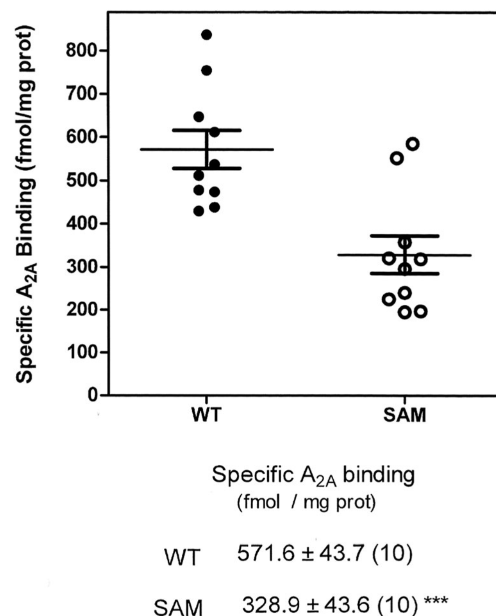

Fig. 1 SAM treatment reduced striatal $A_{2A}R$ ligand binding. The $A_{2A}R$ expression in saline- (WT, $n=10$) and SAM-treated ($n=10$) rats was determined by means of radioligand-binding experiments performed in striatal membrane extracts (see "Materials and methods"). Thus, for each animal, the striatal $A_{2A}R$ content was determined (in triplicate) by displacing the binding of [^{3}H]ZM 241385 (40 nM) to striatal membranes with 5-mM theophylline (specific $A_{2A}R$ binding). The plot shows the mean±SEM for control and SAM-treated rats. $^{***}P<0.001$ compared with non-treated rats. Data were analyzed with Student's *t* test

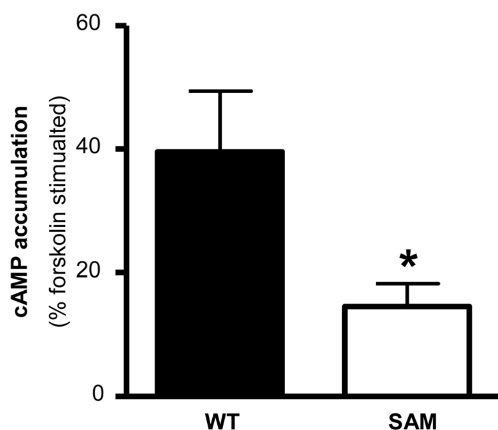

Fig. 2 SAM treatment reduced striatal $A_{2A}R$ function. The striatal $A_{2A}R$ function in saline- (WT) and SAM-treated rats was determined by means of cAMP assay performed in membrane extracts (see "Materials and methods"). Thus, for each animal, the striatal $A_{2A}R$-mediated cAMP accumulation was determined upon incubation with CGS21680 (200 nM) during 30 min. Forskolin-stimulated cAMP was set as 100 %, and *bars* represent the mean±SEM of five animals performed in quadruplicate. $^{*}P<0.05$ compared with saline-treated rats. Data were analyzed with Student's *t* test

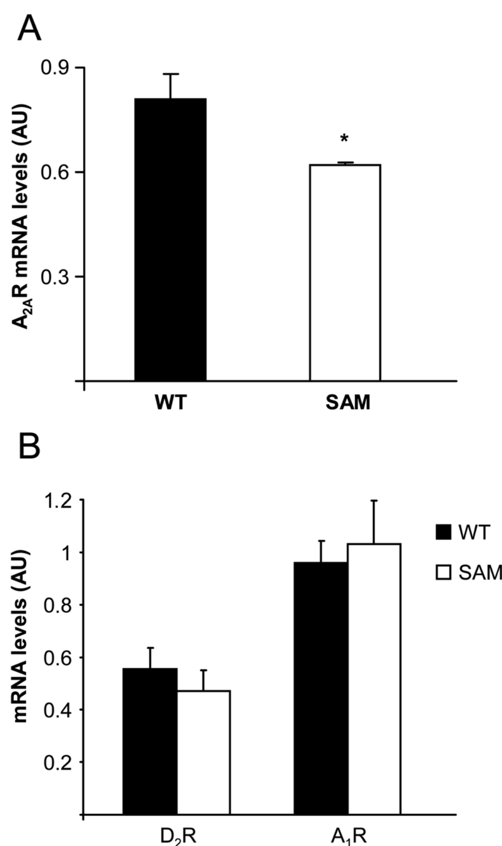

Fig. 3 SAM treatment reduces striatal $A_{2A}R$ mRNA levels. **a** *ADORA2A* and **b** *ADORA1* and *Drd2* mRNA levels were measured in saline- (WT) (n=5) and SAM-treated (n=5) rats by TaqMan PCR. The *ADORA2A, ADORA1*, and *Drd2* mRNA contents were normalized by the amount of the endogenous GUS and expressed as arbitrary units (AU). The plots show the mean±SEM. $^{*}P<0.05$ (Student's *t* test) when compared to saline-treated rats

assessed the $A_{2A}R$ functionality in striatal membranes from saline- and SAM-treated animals by measuring the $A_{2A}R$-mediated cAMP accumulation upon agonist challenge (i.e., CGS21680). Thus, a significant reduction of ~36 % ($P<0.05$) in the $A_{2A}R$-mediated cAMP accumulation in striatal membranes of SAM-treated animals was observed (Fig. 2). Finally, we measured the $A_{2A}R$ mRNA levels in the striatum of both saline- and SAM-treated animals by means of TaqMan PCR. Noteworthy, a significant reduction of ~27 % ($P<0.05$) was observed in the striatal $A_{2A}R$ mRNA content of SAM-treated animals (Fig. 3a), while A_1R and dopamine D_2 receptor (D_2R) mRNA levels remained unchanged (Fig. 3b). Overall, these results clearly suggested that SAM treatment reduced $A_{2A}R$ expression and function in vivo, thus pointing up to the potential use of SAM to reduce $A_{2A}R$ activity in terms of *ADORA2A* repression.

Since gene repression could be accomplished through DNA methylation and SAM constitutes one of the main biological methyl donor molecules involved in DNA methylation [19–21], we next aimed to ascertain if *ADORA2A* down-regulation was achieved throughout a methylation process. To this end, the 5-methylcytosine content in the 5′UTR region of rat $A_{2A}R$ gene was measured using the MassArray platform of SEQUENOM. Interestingly, an increase in the percentage of methylated DNA in several CpG sites was detected in SAM-

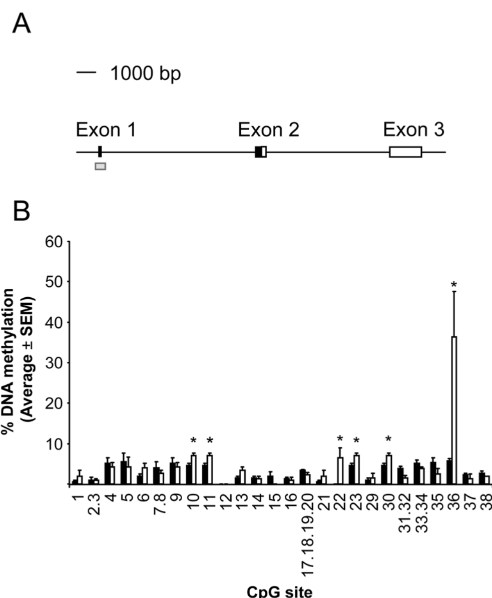

Fig. 4 SAM-mediated striatal *ADORA2A* methylation. **a** Scaled representation of 5′ UTR region of rat *ADORA2A*. Exons 1, 2, and 3 are represented. *White and black boxes* represent coding and non-coding sequences, respectively. The *gray box* represents the locus analyzed. **b** DNA methylation percentage of a locus located in the 5′ UTR region of rat *ADORA2A*. The graph represents the percentage of DNA methylation of each CpG site located in a locus amplified by PCR from saline- (*black bars*; n=5) and SAM-treated (*white bars*; n=5) rats (see "Materials and methods" section). The *x*-axis indicates the data of each CpG site. The plot shows the mean±SEM. $^{*}P<0.05$ (Student's *t* test) when compared to saline-treated rats

treated rats when compared to saline-treated animals (Fig. 4). It is worth mentioning here that some CpG sites within the 5′ UTR region of rat *ADORA2A* could not be quantified by the MassARRAY platform [26]. In addition, some of the CpG sites presented similar degree of methylation since the base-specific cleavage of the in vitro transcription product was not discriminated by the MALDI-TOF analysis (Fig. 4). Nonetheless, SAM treatment mediated the specific methylation of several CpG sites within the striatal *ADORA2A*. Overall, a clear correlation between SAM-mediated methylation and striatal $A_{2A}R$ expression could be established.

It has been proposed that the main cellular localization of striatal $A_{2A}R$ confers an important role on dopaminergic signaling through its interaction with D_2R [27, 28]. As a result of this direct $A_{2A}R$-D_2R interaction [29], antagonists for $A_{2A}R$ have emerged as new targets for non-dopaminergic anti-parkinsonian treatments [30]. Indeed, several clinical trials have shown that the administration of istradefylline (or KW-6002), an $A_{2A}R$ antagonist, ameliorates the dyskinesias induced by chronic levodopa treatment of PD patients [31–38]. Similarly, since $A_{2A}R$ levels have been shown to be increased in the putamen of some PD patients [5–9], it seems likely that *ADORA2A* repression would be an alternative therapy to reduce $A_{2A}R$ activity. Interestingly, several cerebral areas, such as cerebellum, show reduced $A_{2A}R$ levels, a fact that has been correlated with a high percentage of DNA methylation in *ADORA2A*. Therefore, SAM-based treatments would be more effective in those brain regions with low 5-methylcytosine levels in *ADORA2A*, as it occurs in the putamen [15]. In conclusion, based on the restrictive expression of $A_{2A}R$ in the brain and the present results showing that SAM treatment affects striatal $A_{2A}R$ levels, it seems reasonable to consider that SAM may represent a potential co-adjunctive therapy by reducing A_{2A}-mediated D_2R inhibition in the management of PD.

Acknowledgments This work was supported by the Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III (grant CP08/00095 to MB), Ministerio de Economía y Competitividad (grants SAF2011-24779, Consolider-Ingenio CSD2008-00005 and PCIN-2013-019-C03-03 to FC, and BFU2011-23034 to MM), and La Fundació La Marató de TV3 (grants 090330 to MB, and 092331 to MM). Also supported by the Catalan Institution for Research and Advanced Studies (ICREA Academia-2010) is FC. FN and FC belong to the "Neuropharmacology and Pain" accredited research group (Generalitat de Catalunya, 2009 SGR 232). IVM is the recipient of an IDIBELL predoctoral fellowship.

Conflict of interest The authors declare no competing financial interests.

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