Characterization of the levels and G-protein Coupling of Adenosine A\textsubscript{2A} Receptors in Hippocampus and Striatum of transgenic rats overexpressing adenosine A\textsubscript{2A} receptors in the brain

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2015/2016
Characterization of the levels and G-protein Coupling of Adenosine A2A Receptors in Hippocampus and Striatum of transgenic rats overexpressing adenosine A2A receptors in the brain

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Keywords: Adenosine receptors A2A - G protein coupling - cAMP – cognition –

Abstract (Structured as Background, Objectives, Methods, Results, Conclusions)

Background: The transmembrane G-protein coupled adenosine A2A Receptors (A2AR) are one of the main brain targets of adenosine, an homeostatic neuromodulator (Fredholm et al., 2007). Evidence was found of upsurge of A2A R expression, accompanied by coupling and affinity alterations associated to cognitive deficits in ageing (Diógenes et al., 2007; Lopes et al., 1999b) and neurodegeneration, namely Alzheimer’s Disease (Eskelinen et al., 2009). However, it is not known whether A2AR are involved in the development of pathology and aging phenotype; and if the overexpression happens as cause or a consequence of disease.

Objectives: Test the hypothesis that inducing neuronal A2AR overexpressing in the forebrain (CAMkIIa promoter driven human A2A receptor rats) is sufficient to mimic functional and structural changes observed in aged rats.

Methods: Radioligand binding of [3H] ZM241385 (antagonist of A2A R) to perform saturation and competition assays in neuronal membranes from striatum and hippocampus of wild-type (WT) and A2AR over-expression transgenic rats (TG).

Results: Saturation binding for A2AR, showed an increase in Bmáx of the TG compared to WT both in the striatum (1249±79 and 931,6±72,9 fmol/mg, n=3) and the hippocampus (731,2±156,1 fmol/mg versus undetectable levels in WT). The affinity constant (K_D) was similar in the striatum (0,123±0,045 nM) of TG and WT (0,106±0,05 nM); whereas in the hippocampus it was 0,62±0,39 nM for TG (n=3).

Studies of Competition with Gpp(NH)p were performed to investigate the coupling of A2AR to G-proteins. In the striatum, the GTP shift1 was 166% (n=4) in WT and it was superior in TG: 194 (n=4). Analytical comparison showed significant difference (p-value of 0,03). In the hippocampus, TG GTP shift was 211% (n=4), showing no significant difference from the striatum of TG.

Conclusions: The major conclusion of the present study is that over-expression of A2A receptors in the forebrain induces increased coupling of G-protein to A2AR in striatum and hippocampus. This profile is similar to the one found in aged animals (Lopes et al., 1999b), albeit with lower magnitude. This suggests the contribution of other factors, besides over-expression, to the modification of GTP binding profile.

1 The GTP shift was calculated as the ratio of Ki value in the presence of Gpp(NH)p per the Ki value in the absence of Gpp(NH)p which shifts high-affinity G-protein coupled receptors to low-affinity uncoupled receptors.
Introdução: Os receptores transmembranares de adenosina do subtipo A2A (A2AR) acoplados a proteína G constituem um dos principais alvos da adenosina, um neuromodulador homeostático (Fredholm et al., 2007). Estudos determinaram que existe uma associação entre a sobre-expressão de A2AR, alterações do acoplamento e afinidade; e os défices cognitivos da senescência (Diógenes et al., 2007; Lopes et al., 1999b) e neurodegeneração, nomeadamente na doença de Alzheimer (Eskelinen et al., 2009).

No entanto, ainda não está definido se os A2AR estão envolvidos no desenvolvimento de patologia e senescência; e se a sobre-expressão ocorre como causa ou consequência da doença.

Objectivos: Testar a hipótese de que a indução da sobre-expressão neuronal de A2AR no prosencéfalo (promotor CAMKIIa derivado dos humanos em receptor de A2A de ratos) é suficiente para reproduzir as modificações estruturais e funcionais observados nos ratos senescentes.

Métodos: Ensaios de ligação com o radioligando de [3H] ZM241385 (antagonista de A2AR) para ensaios de saturação e competição em membranas neuronais de regiões do estriado e hipocampo de ratos selvagens (WT) e ratos transgénicos que sobre-expressam A2AR com o objectivo de avaliar densidade de receptores e acoplamento a proteínas G.

Resultados: Ensaios de saturação para A2AR, mostram um aumento em Bmáx de TG comparado com WT no estriado (1249±79 and 931,6±72,9 fmol/mg, n=3) bem como no hipocampo (731,2±156,1 fmol/mg versus níveis indetectáveis nos WT). A constante de afinidade (Kd) determinada é semelhante no estriado de TG (0,123±0,0 nM) e WT (0,106±0,05 nM); enquanto que no hipocampo foi 0,62±0,39 nM para TG (n=3). Ensaios de Competição com GppNHp foram realizados para investigar o acoplamento de A2AR a proteína G. No estriado, o GTP shift foi de 166 (n=4) no WT e era superior em TG: 194 (n=4). A comparação analítica dos dados revelou diferença significativa (p-value de 0,03). No hipocampo, o GTP shift foi de 211 (n=4), sem diferença significativa em relação ao estriado dos TG.

Conclusões: A principal conclusão do estudo é de que a sobre-expressão de receptores A2A no prosencéfalo induz o aumento do acoplamento da proteína G de A2A no estriado e hipocampo. Este perfil é semelhante ao encontrado em animais senescentes (Lopes et al., 1999b), ainda que de menor magnitude. Isto sugere a contribuição de outros factores, para além da sobre-expressão, na modificação do perfil de ligação ao GTP.

Abbreviations: A1Rs – Adenosine A1 receptors; A2ARs – Adenosine A2A receptors; CGS 21680 – 2-[4-(2-P-carboxyethyl)phenylamino]-50-N-ethylcarboxamidoadenosine; CGS 15943 – 9-chloro-2-(2-furyl)(1,2,4)triazolo(1,5-c)quinazolin-5-amine; [3H]ZM241385 – 4-[2-[7-[2-3H]Amino-2-(2-furyl)[1,2,4] triazolo[2,3-a][1,3,5]triazin-5-ylamino]-ethyl]phenol; Bmáx: maximum value of binding; GTP- G-protein; KD - affinity constant; Ki; WT- Wild-type Animals; TG- Transgenic Animals
Introduction

Ageing is associated with cognitive decline both in humans and animals. The cognitive impairments observed with age and neurodegenerative diseases are accompanied by structural and functional alterations in the hippocampus, that directly affect neural plasticity (Burke and Barnes, 2006), leading to synaptic dysfunctions and, subsequently, memory deficits (Diogenes et al., 2011).

The present study focuses on the role of adenosine in the realm of neuroprotection. This homeostatic neuromodulator acts predominantly via two known receptors with opposite physiological actions: inhibitory $A_1$ and facilitatory $A_{2A}$ receptors (Ribeiro et al., 1996).

Adenosine $A_{2A}$ Receptors ($A_{2AR}$) are one of the main brain targets of adenosine (Fredholm et al., 2007). $A_{2AR}$ are constitutively activated G-protein coupled-receptors, preferentially expressed by the striatopallidal medium spiny striatal neurons (Blum et al., 2003; Schiffmann and Vanderhaeghen, 1993) but they are also present throughout the brain albeit with a considerably lower density.

However, compelling evidence was found that, upon ageing, there is cortical and hippocampal upsurge of $A_{2AR}$ expression/function and changes in its affinity profile associated to the cognitive deficits observed (Cunha et al., 1995; Lopes et al., 1999b).

Specifically, in the hippocampus of aged rats, $A_{2AR}$ expression is nearly two fold than of young ones (Cunha et al., 1995; Lopes et al., 1999b). Interestingly, other detrimental conditions associated to cognitive impairments, such as hypoxia, diabetes, stress or epilepsy share similar $A_{2AR}$ overactivation (Batalha et al., 2013; Lopes et al., 1999a; Lopes et al., 2011).

Recently, it was described impairment of long-term potentiation (LTP) and hippocampal dependent tasks in an early-aging model, associated with increased $A_{2AR}$ hippocampal expression (Batalha et al., 2013).

The relevance of adenosine in physiological and pathological processes of the nervous system was acknowledged when pioneer studies recognized an inverse relation between the consumption of caffeine, an $A_{2A}$R antagonist, and the risk for development of Parkinson Disease (Ascherio et al., 2001; Ross and Petrovitch, 2001) and Alzheimer’s Disease (Eskelinen et al., 2009; Lindsay et al., 2002; Maia and de Mendonça, 2002). It is now well established that under normal habitual caffeine consumption, the effects exerted in the brain by caffeine depend on its ability to block adenosine A1 and A2A receptors (Fredholm et al., 1999). Moreover, the beneficial impact of A2A receptor deletion or anti-A2A therapeutics in transgenic mouse models of AD-like pathology was recently proven (Laurent et al., 2016; Matos et al., 2012).

Strikingly, recent studies in animal models, showed that it was possible to restore synaptic and cognitive dysfunction by blocking $A_{2AR}$ with the selective antagonist KW6002 (Batalha et al., 2013) orally administered for one month.

This supports an instrumental role of $A_{2AR}$ dysregulation in the genesis of synaptic dysfunction underlying cognitive impairments. However, the mechanism involved or whether $A_{2AR}$ upsurge is sufficient to accelerate hippocampal aging is yet unknown.

The ultimate question that is being addressed is whether the increase in $A_{2AR}$ is either the cause or a consequence for the development of
pathology and aging. To address this, we established a novel transgenic rat strain TGR(CamKIIhA2A), overexpressing A<sub>2A</sub>R under the control of the CamKII promotor. The animals display learning impairments but it remains to be established if this is associated with modifications in the affinity profile of the receptor similar to those found in aged animals in literature.

So I tested this model to determine whether A<sub>2A</sub>R density, affinity for ligand and G-protein coupling is modified as it was found in aged rats. To assess this possibility, the model was tested for A<sub>2A</sub>R’s density using binding experiments of the saturation type and the variation in G protein coupling to A<sub>2A</sub> receptors was determined using binding experiments of the competition type.

Methods

Animals: Animal procedures were performed within the rules of the Portuguese official veterinary department, which complies with European Directive 2010/63/EC and the Portuguese law transposing this Directive (DL 113/2013), and approved by the Instituto de Medicina Molecular Internal Committee and the Portuguese Animal Ethics Committee (Direcção Geral de Veterinária). Environmental conditions were kept constant: food and water ad lib, 21±0.5°C, 60±10% relative humidity, 12 h light/dark cycles. Male rats were killed by decapitation after anesthesia under halothane atmosphere.

Generation and maintenance of transgenic animals: Transgenic rats with an overexpression of adenosine A<sub>2A</sub> receptors (A<sub>2A</sub>R) under the control of the CaMKII promoter, tg(CaMKII-hA2AR), were generated by microinjection of a linearized DNA construct into the male pronucleus of Sprague–Dawley rat zygotes with established methods (Popova et al., 2002). The construct contained a full-length human A<sub>2A</sub> cDNA cloned into an expression vector with the 8.5 kb mouse CaMKIIa promoter (Mayford et al., 1996) and a polyadenylation cassette of bovine growth hormone (Coelho et al., 2014). Wild type (WT) littermates were used as controls.

Genotyping: Transgenic rats were identified by PCR (30 cycles, 58 °C annealing temperature) of their genomic DNA isolated from ear biopsies by the use of the following transgene-specific primers: CaMKII-hA2A and rat Act-B primers as an internal control (Invitrogen, UK, see supplementary Table 1).

Chemicals: 4-[2-[7-[2-3H]-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]-ethyl]phenol ([3H]ZM241385; specific activity, 17 Ci/mmol) was from Tocris Cookson (Bristol, U.K.). Adenosine deaminase (ADA; type VI, 1,803 U/ml, EC 3.5.4.4), 59-guanylylimido diphosphate [Gpp(NH)p], and hemicolinium-3 were from Sigma (St. Louis, MO, U.S.A.). 8-[4-[(2-Aminoethyl)amino]carbonyl methylxophenyl]xanthine (XAC) and CGS21680 were from Research Biochemicals International (Natick, MA, U.S.A.).

Membrane binding experiments

[<sup>3</sup>H] ZM241385 binding studies of the saturation and competition type were performed as previously described (Cunha et al., 1995), using membranes from striatum and hippocampus of wild-type and A2A over-expressive transgenic rats.
Briefly, brains were removed from 12 rats of each genotype and the striata and hippocampus were dissected out at 4°C in sucrose solution (0.32 M) containing 50 mM Tris-HCl, 2 mM EGTA, pH 7.6. The tissue was homogenized in a potter-elvehiem homogenizer at 4°C. The resulting homogenates were centrifuged at 1000 x g for 10 min. at 4°C. The pellets were then resuspended in a solution containing 50 mM Tris-HCl, 2 mM EGTA, pH 7.6.

The tissue was homogenized in a potter-elvehiem homogenizer at 4°C. The resulting homogenates were centrifuged at 1000 x g for 10 min. at 4°C. The pellets were re-centrifuged at 14000 x g for 12 min. at 4°C. The pellets were then resuspended in a solution containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA and 2 U/ml ADA and incubated for 30 min. at 37°C to remove endogenous adenosine. After centrifugation at 14000 x g the pellets were resuspended in the incubation solution (pH 7.4) containing 50 mM Tris-HCl, 2 mM (saturation) or 10 mM (competition) of MgCl₂ and 2 U/ml ADA. Aliquots were frozen and stored at -20 °C for the studies of the saturation type. Fresh samples were used for the studies of the competition type.

In the studies of the saturation type samples were incubated with 7 concentrations of the antagonist [³H]ZM241385 from 0-7nM for 1 h at room temperature. In the studies of the competition type samples were incubated with [³H]ZM 241385 6 nM and 12 known concentrations of A₂AR agonist CGS 21680 from 0-6 µM and with either buffer or Gpp(NH)p 600 µM. This was performed with 40-10 µg of membrane protein for striatum and 300-100 µg for hippocampus in a final volume of 300 µL in an incubation solution of Tris/Mg solution. Non-specific binding of A₂A receptor antagonist [³H]ZM 241385 was measured with A1/A2 receptor antagonist XAC (12 µM). All binding assays were performed in duplicate. The binding reactions were stopped by vacuum filtration. The washing volume was of 5 mL per well in both the experiments of the saturation and the competition type, with the respective incubation buffer maintained at 4°C. The filters were placed in scintillation liquid (Ready Safe, Wallac, Finland) and radioactivity was determined after at least 12 h of incubation. The counting efficiency was of 61%. The protein concentration was determined using the Bio-Rad protein assay based on Bradford dye-binding procedure.

The specific binding was fitted by non-linear regression to a one-site binding equations using the Raphson-Newton method, performed with commercial software (GraphPad San Diego, CA, U.S.A.). Data are the mean ± s.e.m. values (95% confidence interval) of n experiments. A value of p < 0.05 was considered to represent a significant difference.

**Results**

**Binding of [³H]ZM 241385 to the Striatum**

The pharmacological characterization of adenosine A₂A receptors in the striatum has been previously determined for wild-type animals and comparison was made between young adult rats and aged rats (Lopes et al., 1999a, b). In the present study, we characterized the A₂AR in the transgenic overexpressing rats and compared the results to those of wild-type animals.

As it was expected, the Bₘₐₓ. of [³H]ZM 241385 was higher in the transgenic animals 1249±79 fmol/mg than that of wild-type animals 931.6±72.9 fmol/mg. The Kᵦ determined in both animals didn’t show any significant difference, 0.123±0.045 nM for the transgenic animals and 0.106±0.05 nM for wild-type animals, with a p-value of 0.4992 (n=3; Fig.1)
Binding of \[^{3}H\]ZM 241385 to the Hippocampus

The pharmacological characterization of adenosine A\(_{2A}\) receptors in the Hippocampus was determined for the first time in the present study, because the basal expression of these receptors in the hippocampus of wild-type animals is very low.

Again, as it was expected, the B\(_{\text{max}}\) of \[^{3}H\]ZM 241385 displayed the high density of these receptors in the transgenic animals with 731.2±156.1 fmol/mg. The K\(_{D}\) determined was 0.62±0.39 nM which represents a significant difference from the affinity profile of the receptor in the striatum, with a \(p\)-value of 0.0023 (n=3; Fig.1). Saturable binding profile in the hippocampus of wild-type animals was absent.

G protein coupling of adenosine A\(_{2A}\) receptors

We investigated the G protein coupling of A2A receptors using competition binding assays in which we tested the displacement of \[^{3}H\]ZM241385 binding produced by the tested competitor \[^{3}H\]CGS21680, in the absence and in the presence of Gpp(NH)p, which shifts high-affinity G-protein coupled receptors to low-affinity uncoupled receptors.

The GTP shift was calculated as the ratio of K\(_{i}\) value in the presence of Gpp(NH)p per the K\(_{i}\) value in the absence of Gpp(NH)p, in order to ascertain whether over-expression of A\(_{2A}\)R is sufficient to produce change in G-protein coupling similar to that observed in aged rats.

\[
GTP\text{shift} \equiv \frac{K_{i,\text{Gpp(NH)p}}}{K_{i,\text{Gpp(NH)p}}},
\]

G protein coupling of adenosine A\(_{2A}\) receptors in the Striatum

In the wild-type animals, the K\(_{i}\) obtained for the samples without Gpp treatment was 21.07±3.6 nM and in those with Gpp
it was 34.93±5.3 nM. Therefore, the GTPshift calculated is 166%.

In the transgenic animals the Ki obtained for the samples without Gpp treatment was 16.27±3 nM and in those with Gpp it was 31.59±5.3 nM. Therefore, the GTPshift calculated is 194%.

The statistical analysis determined a significant difference in the comparison of A2AR’s affinity in transgenic and wild-type, with a p-value of 0.0314 (n=4; Fig.2).

G protein coupling of adenosine A2A receptors in the Hippocampus

The Ki obtained for the samples without Gpp treatment was 49.58±14.6 nM and in those with Gpp it was 104.5±30.4 nM. Therefore, the GTPshift calculated is 211% (Fig. 3)

Statistical analysis showed significant difference between the affinity of A2AR in the striatum and in the hippocampus, with a p-value of 0.0134 (n=4).
Discussion

The major conclusion derived for the present study is that over-expression of A$_{2A}$ receptors in the brain is able to produce modification of G-protein binding profile with increased coupling to the receptor in the brain regions of Striatum and Hippocampus.

The previous conclusion was derived from the observation of significant difference in the G-protein binding profile between wild-type and transgenic animals. In the wild-type animals, decoupling of G-protein produced 166% fold-change in the displacement of A$_{2A}$R ligand whereas in the transgenic animals it produced a 194% fold-change. Analytical comparison showed significant difference (p-value of 0.03) between these values.

The relevance of these results is related to the observation of functional and structural changes in A$_{2A}$R of aged rats with increased density and G-protein coupling, being clear so far that these changes in adenosine receptors are paralleled by a detriment in behavioral patterns and cognitive function. Consequently, the increased G-protein binding produced exclusively by transgenic over-expression of A$_{2A}$R, undeniably confirms the influence of over-expression in the increased G-protein coupling to the A$_{2A}$R observed in the aged groups studied in the literature (Lopes et al., 1999b).

However, the effect of decoupling of G-protein in the aged groups studied in the literature produced 321% fold-change in the displacement of A$_{2A}$R ligand in comparison to the 199% observed in the transgenic animals. Therefore, age-related change in the G-protein binding to A$_{2A}$ receptor, produces greater coupling profile than merely transgenic over-expression of the receptor.

These results suggest the contribution of multiple factors to the modification of G-protein binding profile namely other intracellular pathways or other neuromodulatory systems and, consequently, the pathological phenotype of diseased animals.

To this point, it is relevant to emphasize that even though the over-expression produced only modest G-protein binding profile modification, its effects were sufficient to produce pathological phenotype, established by the working memory deficits revealed by the transgenic rat strain, TGR, overexpressing adenosine A2ARs (Giménez-Llort et al., 2007). The reasons appointed for phenomenon are that adenosine binding to a membrane with high density of A$_{2A}$ receptors produces the same physiological neuronal modulation as a membrane with modest density of A$_{2A}$ receptors but a strong G-protein binding profile, as it is observed in the aged rats previously studied (see Lopes et al., 1999b).

Finally, another major conclusion derived from the present study was the determination of A$_{2A}$ receptor affinity in the Hippocampus. Our knowledge about the role of A2ARs in modulating neuronal activity is more limited. This is mainly because most of the studies have focused in the basal ganglia where A2ARs are by far more abundant because of their ‘abnormal’ large expression in the medium spiny neurons of the indirect pathway. Thus, the study of these A2ARs, which have a particular density and subcellular localization in this particular set of neurons, might not be representative of the more general role of A2ARs in the most regions of the brain (Cunha, 2005).
In this project comparison has been made between affinity of $A_{2A}$R in transgenic animals in the striatum and in the hippocampus and showed significant difference between this groups with a $p$-value of 0.0134 for fitting. These results show us that affinity for the $A_{2A}$R differs throughout the brain. Currently, no explanation for this phenomenon has been conclusively ascertained, however, it is believed that these results express a property of $A_{2A}$R that has been recently discovered, the heterodimerization with $A_1$R (Ciruela et al., 2006). This could explain the differences in affinity assuming that in the various regions of the brain, there are different proportions of these heterodimers.

**FUNDING AND ACKNOWLEDGEMENTS**

Marta Leal Bento was supported by a grant from Gulbenkian/ FMUL (Faculty of Medicine of the University of Lisbon), having been attributed the classification of “Very Good” and funded with 3,000,00 € (20130009/PEC/BG) in the context of GAPIC Project, for undergraduates.

Luisa Lopes (IMM, Portugal; http://imm.fm.ul.pt/web/imm/llopes) is a Principal Investigator@IMM and obtained a position as an FCT Researcher (Investigador FCT). The authors acknowledge João Baião for technical assistance in the animal house and in the radioactive material laboratory.

**Author contributions**

Marta Leal Bento has written the draft and performed all the experimental work under supervision from Joana E. Coelho and Vânia L. Batalha. Marta Leal Bento was trained on the subject of Radiation Safety by IMM. Luisa V. Lopes and Marta Bento designed the experiments, discussed the experimental findings and revised the manuscript.

**Conflict of interest**

There are no known conflicts of interest associated with this project. The manuscript has been read and approved by all named authors.

**Financial conflict of interest**

There has been no significant financial support for this work that could have influenced its outcome.

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Anexes:

1. Primers used for genotyping and qPCR.

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