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Increased striatal adenosine A_{2A} receptor levels is an early event in Parkinson's disease-related pathology and it is potentially regulated by miR-34b

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ABSTRACT

Adenosine A_{2A} receptor (A_{2A}R) is a G-protein coupled receptor that stimulates adenylyl cyclase activity. In the brain, A_{2A}Rs are found highly enriched in striatal GABAergic medium spiny neurons, related to the control of voluntary movement. Pharmacological modulation of A_{2A}Rs is particularly useful in Parkinson's disease (PD) due to their property of antagonizing dopamine D_2 receptor activity. Increases in A_{2A}R levels have been described in PD patients showing an important loss of dopaminergic denervation markers, but no data have been reported about A_{2A}R levels in incidental PD brains. In the present report, we show that increased A_{2A}Rs protein levels were also detected in the putamen of incidental PD cases (Braak PD stages 1–2) with respect to age-matched controls. By contrast, A_{2A}Rs mRNA levels remained unchanged, suggesting that posttranslational mechanisms could be involved in the regulation of A_{2A}Rs. It has been described how miR-34b/c downregulation is an early event in PD cases. We found that miR-34b levels are also significantly reduced in the putamen of incidental PD cases and along disease progression. Given that 3′UTR of A_{2A}R contains a predicted target site for miR-34b, the potential role of this miRNA in protein A_{2A}R levels was assessed. *In vitro* studies revealed that endogenous A_{2A}R protein levels increased when miR-34b function was blocked using a specific anti-miR-34b. Moreover, using a luciferase reporter assay with point mutations in a miR-34b predicted binding site within the 3'UTR region of A_{2A}R mRNA abolished the effect of the miRNA using a miR-34b mimic. In addition, we showed a reduced percentage of DNA methylation in the 5'UTR region of *ADORA2A* in advanced PD cases. Overall, these findings reveal that increased A_{2A}R protein levels occur in asymptomatic PD patients and provide new insights into the molecular mechanisms underlying A_{2A}R expression levels along the progression of this neurodegenerative disease.

Introduction

Parkinson's disease (PD) is a human neurodegenerative disease characterized by the loss of dopaminergic neurons in the *substantia nigra pars compacta* and the presence of α-synuclein-containing intraneuronal inclusions called Lewy bodies and aberrant Lewy neurites (Braak et al., 2003). PD is clinically manifested by the presence of motor disturbances such as resting tremor, bradykinesia, rigidity, and postural instability. According to Braak et al. (2003) α-synuclein inclusions are first found in the medulla oblongata (stages 1–2), then the midbrain and amygdala (stages 3–4) and finally the cerebral cortex (stages 5–6). Motor symptomatology appears after the loss of dopaminergic neuronal cells in the *substantia nigra pars compacta* (more than 60%) at late stage 3 or 4 of Braak. Incidental or pre-motor PD corresponds to those cases with Lewy bodies or neurites in the brain stem without any motor disturbance (Jellinger, 2004; Saito et al., 2004).

Adenosine is an endogenous purine nucleoside that mediates a wide variety of physiological functions by interaction with four G-protein-coupled receptors which modulate adenylate cyclase activity: A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2001). In the central nervous system

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(CNS), A_1Rs are associated with neuroprotective processes and they are upregulated in human neurodegenerative diseases with abnormal protein aggregates (Albasanz et al., 2007, 2008; Angulo et al., 2003; Perez-Buira et al., 2007; Rodríguez et al., 2006). By contrast, A_{2A}Rs activity is related to the modulation of glutamate release and it is associated with the outcome of cerebral injury as well as the development of Aβ-induced synaptotoxicity (Canas et al., 2009; Cunha, 2005; Stone et al., 2009). The brain region most enriched in A_{2A}Rs is the *striatum*, in which A_{2A}Rs are largely restricted to GABAergic spiny medium neurons of the indirect pathway, projecting from the caudate-putamen to the external *globus pallidus* and also expressing dopamine D_2 receptors (D_2Rs) (Fuxe et al., 2007). It has been reported that A_{2A}R expression levels are upregulated in the putamen and in peripheral blood cells from PD patients with levodopa-induced dyskinesias, and that these levels correlate with the severity of the disease (Calon et al., 2004; Casetta et al., 2013; Varani et al., 2010). Moreover, PET studies have confirmed striatal A_{2A}R upregulation in PD (Mishina et al., 2011; Ramlackhansingh et al., 2011). However, it needs to be clarified whether A_{2A}R upregulation occurs in early Braak PD stages (incidental PD).

In the last few years the study of the transcriptional mechanisms of A_{2A}R gene (*ADORA2A*) regulation has demonstrated that DNA methylation plays a role in the baseline A_{2A}R expression levels in the brain (Buira et al., 2010a, 2010b). DNA methylation is an epigenetic mechanism associated with gene repression (Jones, 2012). Interestingly, an increase in the DNA methylation percentage in *ADORA2A* gene has been attributed to reduced A_{2A}R protein levels in Huntington's disease (HD) and in a subgroup of schizophrenia patients (Villar-Menéndez et al., 2013, 2014).

Therefore, based on these findings, our study was designed to clarify whether A_{2A}R upregulation was an early event in incidental PD patients, and to elucidate potential molecular mechanisms playing a role in pathological *ADORA2A* expression. In this sense, not only has the role of DNA methylation been studied, but also the putative role of microRNAs (miRNAs) in the control of A_{2A}R protein levels. miRNAs are small non-coding RNAs, 21–24 nucleotides in length, that act as post-transcriptional regulators, binding to specific binding sites located in the 3'-untranslated region (3'UTR) of mRNAs to inhibit their translation by promoting mRNA degradation or by blocking ribosomal activity (Lagos-Quintana et al., 2001). A previous study described how reduced miR-34b/c levels were an early event in PD (Miñones-Moyano et al., 2011). Given that the 3'UTR region of A_{2A}R mRNA shows predicted binding sites for miR-34b, the present study was designed to probe more deeply into the putative relationship between miR-34b and A_{2A}R protein levels.

Material and methods

Human brain samples

Tissue samples were provided by the Neurological Tissue Bank, Hospital Clínic de Barcelona, and the Institute of Neuropathology, HUB-ICO-IDIBELL Brain Bank. The donation and obtaining of samples from the CNS are regulated by the ethics committees of the two institutions. The sample processing followed the rules of the European Brain Bank Network: BrainNet Europe II (BNEII). All the samples are protected in terms of individual donor identification following the BNEII laws. The brains were maintained half in formalin for morphological and immunohistochemical studies, while the other half was processed in coronal sections to be frozen at −80 °C and made available for biochemical studies. The neuropathological exams were performed in all cases on twenty sections of brain, both *cerebellum* and brainstem. For a first approximation towards a neuropathological diagnosis of the disease, sections were stained with haematoxylin and eosin, luxol fast blue-Klüver Barrera, and subjected to immunohistochemistry for Tau, β-A4 Amyloid, α-Synuclein, α-Bcristalin, and Ubiquitin. Brains from individuals without neurological history or neuropathological lesions after the standard exam were used as control group samples. The neuropathological diagnoses were made according to well-established criteria for PD (Braak et al., 2003). All cases analyzed are summarized in Table 1.

Quantitative DNA methylation analysis

The percentage of DNA methylation was evaluated in three loci within the 5′UTR of *ADORA2A* gene, previously reported as being relevant for its transcriptional regulation (Buira et al., 2010b). DNA purification, bisulfite treatment, and quantitative DNA methylation analysis were performed with the MassArray platform of SEQUENOM as described (Buira et al., 2010b). The primers used for PCR amplification were the following: A_{2A}R-10069 (PCR 1), A_{2A}R-8973 (PCR 2), and A_{2A}R-7883 (PCR 3) (Fig. 2A) (Buira et al., 2010b).

Cell culture

Human neuroblastoma SH-SY5Y cell line was maintained in DMEM medium (Invitrogen, El Prat de Llobregat, Spain) (ECACC number: 94030304) supplemented with 10% fetal bovine serum. Depending on experimental conditions, cells were maintained in culture with the addition of 10 μM of retinoic acid (RA) in the growth medium for 96 h (Sigma, Madrid, Spain). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

Anti-miR-34b transient transfection

SH-SY5Y cells were plated on 12-well dishes at a concentration of 60,000 cells/well and cultured overnight before transfection. The following day, cells were treated with 10 μM RA for 96 h. Cells were transfected with 50 nM anti-miR-34b (Exiqon, cat. no. 410215-00) in OptiMEM (Invitrogen) using Lipofectamine™ 2000 (Invitrogen) and following the instructions of the manufacturer. After 5 h of transfection, the medium was replaced with fresh medium. Cells were maintained for 48 h in the presence of RA. As a negative control, scrambled anti-miRNA was used (Exiqon, cat. no. 199004-00).

miRNA luciferase reporter assay

A vector pMirTarget containing wild-type A_{2A}R 3'UTR (Origene, Rockville, MD, USA) was used to measure luciferase activity. Point mutations were introduced into a predicted miR-34b binding site located in the A_{2A}R 3′UTR using the QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's instructions. The primers used were: Forward, 5′-ACCCAGTGAG AGGCCTTGTCTATGTCCGTTTCTTCTAAAGGGAATGT-3′, and Reverse, 5′-ACATTCCCTTTAGAAGAAACGGACATAGACAAGGCCTCTCACTGGGT-3′. The sequence of the mutated vector was verified by sequence analysis (Macrogen, Amsterdam, The Netherlands).

One microgram of A_{2A}R 3'UTR pMirTarget or mutated vectors was cotransfected into non-RA-treated SH-SY5Y cells with the 50 nM of mimic miR-34b (Ambion, cat. no. MC10743). After 48 h, cells were lysed using the reporter lysis buffer (Promega, Madrid, Spain) and luciferase was measured in a Fluostar Optima lector (BMG Labtech, Madrid, Spain). As pMirTarget vectors co-express the red fluorescence protein (RFP), the fluorescence was measured in the same lysates and used to normalize the luciferase activity results.

RNA isolation

Total RNA was extracted from 50 mg of human putamen with the RNeasy Lipid Tissue Mini kit (Qiagen, Madrid, Spain) following the protocol provided by the manufacturer. The concentration of each sample was measured with a Nanodrop 1000. RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent Technologies). Only RNA samples with RIN > 6 were analyzed.