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Expression levels of adenosine receptors in hippocampus and frontal cortex in argyrophilic grain disease

S. Perez-Buira [a], M. Barrachina [a], A. Rodriguez [a], J.L. Albasanz [b], M. Martín [b], I. Ferrer [a,*]

[a] *Institut de Neuropatologia, Servei Anatomia Patològica, IDIBELL-Hospital Universitari de Bellvitge, Facultad de Medicina, Universitat de Barcelona, carrer Feixa Llarga sn, 08907 Hospitalet de Llobregat, Spain*
[b] *Departamento de Química Inorgánica, Orgánica y Bioquímica, Facultad de Químicas, Centro Regional de Investigaciones Biomédicas, Universidad de Castilla-La Mancha, Ciudad Real, Spain*

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Abstract

Expression of adenosine receptors of the A1, A2A and A2B type has been examined in the post-mortem frontal cortex and hippocampus in argyrophilic grain disease (AGD), a tauopathy affecting the hippocampus but usually not the frontal cortex, in an attempt to learn about the modulation of the adenosine pathway in this disorder. Significant increased levels of A1, but not of A2A and A2B, have been observed in AGD in the hippocampus but not in the frontal cortex, when compared with age-matched controls. This is accompanied by increased levels of adenylyl cyclase (AC), an effector of A1, and by increased (although not significant) percentage of inhibition of forskolin-stimulated AC by the A1 agonist cyclohexyladenosine in the hippocampus in AGD. These findings indicate sensitization of A1/AC in the hippocampus in AGD, and support a putative activation of the A1/AC pathway that may facilitate protection of this preferentially involved region in AGD.

Keywords: Argyrophilic grain disease; Adenosine receptors; Adenylyl cyclase

Argyrophilic grain disease (AGD) is a late-onset neurodegenerative disease, producing cognitive impairment and dementia, which is morphologically characterized by the presence of abundant spindle-shaped argyrophilic grains in neuronal processes and coiled bodies in oligodentrocytes, especially in the entorhinal and perirhinal cortices, CA1 region of hippocampus, amygdala and other regions of the limbic system. The neocortex and, particularly, the frontal cortex, are not morphologically affected [5,14,19]. The main biochemical abnormality is the deposition of hyper-phosphorylated tau 4R in the cytoplasm of neurons (usually in the form of pre-tangles) and in the neuropil grains, as well as in oligodendrocytes and certain astrocytes in vulnerable brain regions [21,22,25]. Hyper-phosphorylation of tau is accompanied by selective over-expression of tau kinases in sensitive cell populations [11]. The reasons for cellular degeneration are not known but it may be assumed that injuring mechanisms are combined with compensatory responses to reduce cell damage.

Adenosine is involved in the regulation of different metabolic processes under differing physiological and pathological conditions and mediates its function through the adenosine receptors (ARs) [10,16,23]. A1 and A3 mediate inhibition of adenylyl cyclase (AC) activity through Gαi-proteins, and A2A and A2B mediate stimulation of AC activity through Gαs-proteins [13]. In addition, stimulation of A1 activates phospholipase C, phospholipase D and several types of K^+ channels, and inhibits Ca^{2+} currents [16]. A1 type receptors are found in several tissues [9], but they are enriched in the CNS, where they are mostly expressed in the cerebral cortex, hippocampus, cerebellum, thalamus and brain stem. In the brain, adenosine modulates neuronal activity by decreasing pre-synaptic release of various neurotransmitters [3,12,23]. The most dramatic inhibitory actions are on the glutamatergic system [10].

Based on these findings, and in an attempt to learn about adenosine receptors in AGD, the present study used western blotting to analyze the expression of ARs in the frontal cortex and hippocampus in AGD. Protein studies were accompanied by *in vitro* assays of AC activity using A1 agonists.

Brain samples were obtained from the Institute of Neuropathology brain bank following the guidelines of the local ethics committee. The brains of five patients with AGD (three

* Corresponding author. Tel.: +34 93 260 7452; fax: +34 93 260 7503.
E-mail address: 8082ifa@comb.es (I. Ferrer).

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Table 1
Summary of cases

Case	Age	Gender	p-m delay	Diagnosis
1	62	F	4	Control
2	63	M	17	Control
3	46	F	20	Control
4	73	F	5	Control
5	87	F	5	AGD
6	64	F	9	AGD + ADIII
7	77	F	3	AGD + ADIV
8	72	F	10	AGD
9	65	F	4	Control
10	79	M	7	Control
11	73	F	7	Control
12	71	F	20	AGD

F: female; M: male; p-m delay: post-mortem delay (in hours); AGD: argyrophilic grain disease; AD: Alzheimer's disease stage of Braak; control: no neurological and metabolic disease, and no neuropathological findings.

pure AGD and two AGD with Alzheimer's disease stage III and IV) and four age-matched controls were obtained from 3 to 20 h after death and prepared for morphological and biochemical studies. Age-matched controls ($n = 7$) had no neurological or metabolic abnormalities, and the neuropathological examination was strictly normal including lack of neurofibrillary tangles and amyloid plaques. A summary of cases is found in Table 1. At autopsy, one hemisphere was cut into coronal sections, rapidly frozen on dry ice and stored at −80 °C until use. The other hemisphere was fixed in 4% buffered formalin for 2–3 weeks, and selected samples were embedded in paraffin and processed for morphological study. In addition, samples of the frontal cortex (area 8) and hippocampus were fixed in 4% paraformaldehyde for 24 h, cryoprotected with 30% saccharose, and frozen.

Cryostat sections, 15-μm thick, were processed free-floating. Rabbit polyclonal antibodies to A1 (Oncogene, Barcelona, Spain), A2A (Chemicon, Millipore, Barcelona, Spain) and A2B (Abcam, Cambride, UK) were used at dilutions of 1:1000, 1:500 and 1:500, respectively. The peroxidase reaction was visualized with NH_4NiSO_4 (0.05 M) in phosphate buffer (0.1 M), 0.05% diaminobenzidine, NH_4Cl and 0.01% hydrogen peroxide (dark blue precipitate). Some sections were incubated without the primary antibody. No immunoreactivity was found in these samples.

Immunohistochemistry disclosed A1, A2A and A2B immunostaining in the frontal cortex and hippocampus restricted to neurons, in control and diseased cases. No evidence of immunoreaction in grains was noted in AGD (Fig. 1).

Plasma membrane extracts of the frontal cortex (area 8) and hippocampus were obtained for study. Tissue samples were homogenized in a buffer containing 20 mM Hepes, 0.25 M sucrose, 0.3 mM PMSF, 1 mM DTT, 1 mM EGTA, and 1 mM MgCl2 (pH 7.4), and centrifuged at $600 \times g$ for 10 min. The supernatants were centrifuged at $48{,}000 \times g$ for 20 min. The pellets were re-suspended in a buffer containing 20 mM Hepes, 0.3 mM PMSF and 1 mM DTT (pH 7.4), and centrifuged in the same way. Membrane-enriched pellets were re-suspended in the same buffer and stored at −80 °C until use. Protein concentration was measured with the BCA method using bovine serum albumin (BSA) as a standard. For Western blots, 10 μg of protein was mixed with loading buffer containing 0.125 M Tris (pH 6.8), 20% glycerol, 10% β-mercaptoethanol, 4% SDS and 0.002% bromophenol blue. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (7.5–12% SDS-PAGE) was carried out using a mini-protean system (Bio-Rad, Madrid, Spain) with molecular weight standards. Proteins were transferred to nitrocellulose membranes which were washed with TTBS containing 10 mM Tris–HCl (pH 7.4), 140 mM NaCl and 0.1% Tween-20, blocked with TTBS containing 5% skimmed milk. Then the membranes were incubated with one of the primary antibodies at 4 °C overnight. The rabbit polyclonal anti-A1 (Oncogene) was used at a dilution of 1:1000; the rabbit polyclonal anti-A2A (Chemicon) was used at a dilution 1:500; the rabbit polyclonal anti-A2B (Abcam) was used diluted 1:500; and the rabbit polyclonal anti-AC1 (Santa Cruz Biotechnology, Madrid, Spain) was used at a dilution of 1:1000. After rinsing, the membranes were incubated with the corresponding anti-rabbit secondary antibody (Dako, Madrid, Spain) at a dilution

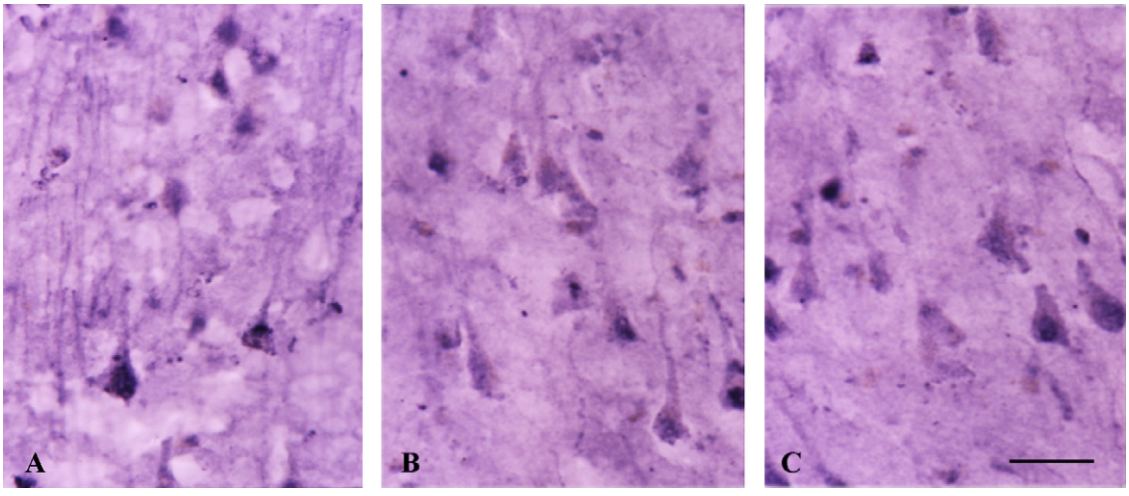

Fig. 1. Immunoreactivity to A1 (A), A2A (B) and A2B (C) in the hippocampus in AGD brain. Immunoreactivity is found in neurons but no evidence of immunostaining occurs in neuropil grains. Thick section processed free-floating. Bar = 25 μm.