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A_{2A} adenosine receptor over-expression correlates with motor symptoms in Parkinson's disease

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ABSTRACT

Adenosine receptors (ARs) are seven trans-membrane domain G-protein coupled receptors named A_1, A_{2A}, A_{2B} and A_3. Adenosine, the main agonist, acting on these receptors exerts a broad spectrum of physiological and pathological functions. Adenosine production increases dramatically when there is a discrepancy between the rates of ATP synthesis and ATP utilization, for example when work load is markedly enhances or when the supply of oxygen and glucose is limiting as in ischemia. Adenosine receptors are expressed in all body tissues with different concentrations and functions. One of the most important role is explain in the regulation of inflammatory function, a field where A_{2A}AR is an important player.

Parkinson's disease (PD) is a pathology with a complex etiology, involving both genetic and environmental factors. The cardinal signs of PD relate to motor dysfunction, psychiatric and dysautonomic symptoms. PD is characterized by prominent loss of dopaminergic neurons in the substantia nigra pars compacta in relatively early stages of the disease, depletion of striatal dopamine, and the presence of intraneuronal inclusions called Lewy bodies. Current knowledge highlight the important role of inflammation in Parkinson's disease. The central nervous system was supposed to be an immune privileged site, in which immune cells of the periphery could not enter or rarely entered. Today we know that peripheral immune responses can trigger inflammation and exacerbation of central nervous system degeneration in several neurodegenerative diseases. When cytokines, such as tumor necrosis factor (TNF)-α, are secreted by activated glia in the brain or are present in circulating blood, permeability of the blood brain barrier is increased.

The primary aim of this study was to investigate the expression, affinity and density of adenosine receptors in lymphocytes and neutrophils of PD patients compared to healthy subjects. This study revealed a specific A_{2A}AR alteration correlating with disease severity: patients with higher A_{2A}AR density and lower affinity were more likely to exhibit motor complications. An increase in A_{2A}AR density in putamen patients was found, an alteration that mirrors a similar up-regulation in human peripheral blood cells.

Moreover, how expected, we measured high levels of adenosine and TNF-α in plasma of PD patients. Interesting we found out a statistically significant linear correlation among the A_{2A}AR density and TNF-α levels. Elevated levels of TNF-α in PD brains amplify and sustain the neuroinflammation leading to dopaminergic neurons destruction. Moreover, several studies highlight a close relation between TNF-α release and A_{2A}AR.

To shed some light on the functional adenosine-dopamine interaction, we examined the effects of well-known A_{2A}AR agonists and antagonists on dopamine uptake in the rat adrenal pheochromocytoma cell line after differentiation into a neuronal phenotype by nerve growth factor. Our results show that A_{2A}AR antagonists decreased dopamine uptake, and an opposite effect was mediated by A_{2A}AR agonists.

In conclusion our data prove the double importance of A_{2A}AR in Parkinson's disease: a biomarker useful for diagnose and a potential therapeutic target for PD.

LIST OF ABBREVIATION

- **ADP**: adenosine di-phosphate
- **AMP**: adenosine mono-phosphate
- **ATP**: adenosine tri-phosphate
- **CCPA**: 2-Chloro-N_6-cyclopentyladenosine
- **CGS 21680**: 4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin 2-yl]amino]ethyl]benzenepropanoic acid hydrochloride
- **CHO**: chinese hamster ovary
- **Cl-IB MECA**: 2-Chloro-N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide
- **CNS:** central nervous system
- **COX**: cyclooxygenase
- **CPA**: N_6-cyclopentyladenosine
- **CREB**: cAMP response element-binding protein
- **DA**: dopamine
- **ENTPD**: ectonucleoside triphosphate diphosphohydrolase
- **HIF**: hypoxia inducible factor
- **IB-MECA**: 1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide
- **IL**: interleukine
- **KO**: knock out
- **LB**: Lewy body
- **LPS**: lypopolisaccaride
- **MAO**: monoamino oxidase
- **MPTP**: 1-metil 4-fenil 1,2,3,6-tetraidro-piridina
- **NECA**: 5'-N-ethylcarboxamido adenosine
- **NF-kB**: nuclear factor kB
- **NK cells**: natural killer
- **NOS**: nitrous oxide systems
- **PBS**: phosphate buffered saline
- **PC12**: pheochromocytoma cell
- **PD81723**: 2-Amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl) phenyl] methanone
- **PGE**: prostaglandin
- **PKA**: protein kinase A
- **PLC**: phospolipase C
- **R-PIA**: N^6-(R-phenylisoprophyl) adenosine
- **SCH 58261**: 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo [1,5-*c*]pyrimidin-5-amine
- **Tgf**: tumor growth factor
- **TNF**: tumor necrosis factor
- **VEGF**: vascular endothelium growth factor
- **WT**: wild type
- **ZM 241385**: 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol

CHAPTER I
INTRODUCTION

INDRODUCTION

1. Adenosine signaling: the beginning

Purines appear to be the most ancient chemical cellular messenger in animals and plants. The purinergic signaling system employs extracellular purines and pyrimidines as signaling molecules both released from living cells. These transmitters act upon target cells through activation of two classes of receptors, the metabotropic P1 receptors to adenosine, and nucleotide receptors of the P2 family, which is subdivided into P2Y metabotropic and P2X ionotropic sub-classes [1].

Adenosine is present ubiquitously and its receptors are widely distributed through the body exerting a broad spectrum of physiological and pathophysiological functions [2]. Data from techniques of molecular biology suggest that P2X ion channel receptors appeared early in evolution while G-protein coupled P1 and P2Y receptors were introduced at the same time or later [1].

The first evidence of adenosine effect date back to eighty years ago. In the University of Cambridge, A.N. Drury and A. Szent-Györgyi, begin their study of a unknown substance extract from bullock's heart muscle. They chose the heart like study model because "the heart, therefore, is an admirable test object for any substance which may be suppose to influence cell activity". The injection of this substance into a dog or guinea pig caused bradycardia. After exclusion of vagus nerve involvement and influence of inorganic component used in the protein extraction, they wrote "we are strongly of the opinion, as a result of these observations, than our substance is identical with adenylic acid, and we shall refer in this paper to the substance isolated by us as adenylic acid". Adenosine prepared from yeast nucleic acid has an identical action to adenylic acid. The lower arterial pressure was due in part to the cardiac slowing and in part to a general arterial dilatation [3].

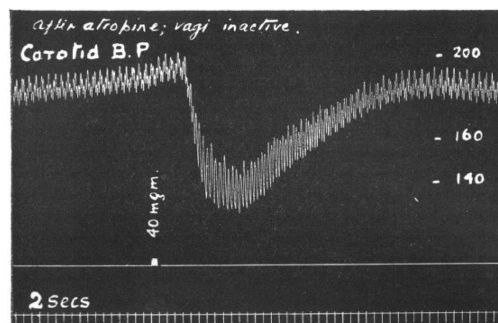


Fig.1 Influence of injection of 40 mg adenosine into femoral vein [3].

Two years later Bennet and Drury confirmed the previous experiment on the heart and came in light the effect of adenosine on others guinea pig body tissues: uterus constriction, local accumulation of leucocytes, considerable fall of temperature after subcutaneous injection [4].

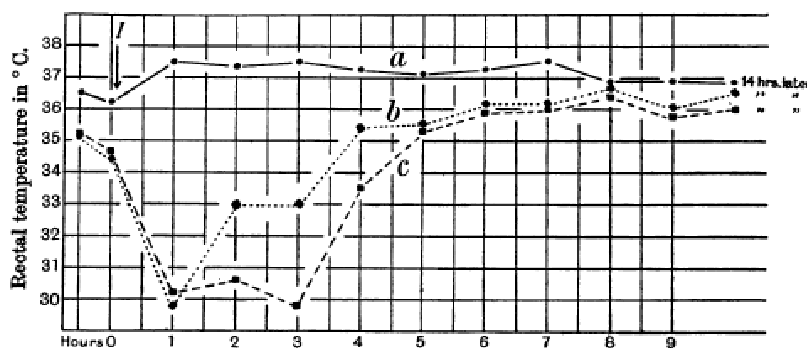


Fig. 2 Influence of subcutaneous injection of adenosine upon rectal temperature of the guinea-pig. a) Injection of saline, b) and c) injection of 50 mg and 100 mg of adenosine [4].

Severity of trauma, magnitude of the loss of tissue adenine nucleotides and the release of breakdown products correlated with protective effects and vasodilatation, anticoagulatory effect, energy transfer suppose to be due to adenosine nucleotides enter cells. Bennet and Drury studied that a lack of energy owing to hypoxia caused the breakdown of myocardial adenine nucleotides able to cause coronary vasodilatation: increasing oxygen supply to an energy-depleted tissue, subsequently limiting its own formation like a classical tenet of homeostasis and negative feedback [5].

From other study on heart it was understood that methylxanthines acted as adenosine antagonists [6]. From 1960 synthesize of more stable analogues started yielding the adenosine analogue 2-chloroadenosine, R-PIA and NECA [7]. The different effects of these compounds away the idea of a therapeutic benefit but helping to define the subtypes of adenosine receptors (a concept become clear in 1970s).

For many years it was though that the only source of extracellular ATP acting on purinoceptors was damaged or dying cells. It is now recognized that ATP release from healty cells is a physiological mechanism in response to shear stress, stretch or osmotic swelling, hypoxia or stimulation by various agents [8].

The concept of purinergic signaling system was first proposed by Geoffrey Burnstock in 1970, when proposed ATP like a neurotransmitter [9]. In 1978 Burnstock made the important suggestion that there exist a family of receptors called purinergic receptors

that can be subgrouped into two subclasses, P1 and P2 [10]. This classification was based on the selective potency of ATP, ADP, AMP and adenosine, the effectiveness of methylxanthines as antagonists and mediation or not by adenylate cyclase [11]. This hypothesis met considerable resistance because ATP had been established as intracellular energy source and it was thought that such a ubiquitous molecule was unlikely to be involved in selective extracellular signaling [8]. The *Purinergic Hypothesis* included the presence of purinoceptors and a few years later Burnstock proposed a basis for distinguish P1 (adenosine) from P2 (ATP/ADP) receptors and in 1985 distinguished two type of P2 purinoceptor, P2X and P2Y [12]. In 1990s with studies of transduction mechanism and cloning of P2X and P2Y it was proposed that there were two families of P2 receptors, P2X ionotropic ligand-gated ion channel receptors and P2Y metabotropic G-protein-coupled receptors.

A classification into A_1 and A_2 adenosine receptor was proposed in 1979 [13] along with the A_{2A} and A_{2B} nomenclature for the two cloned receptors showing considerable sequence homology and similar signal transduction mechanism, distinguished by pharmacological criteria [14].

2. Adenosine signaling: current knowledge

<u>ADENOSINE</u>

Adenosine is the main agonist at the P1 receptor class and in addition the adenosine metabolite inosine can activate at least some of the receptors [15]. The concentration of adenosine in the extracellular compartment is the consequence of many biological processes, including extracellular adenosine production from intracellular sources, transport, and metabolism to inosine or AMP. ATP can be release from vescicles with a "kiss and run" mechanism (vescicles open and close transiently), from the lysosome by exocytosis or by an uncontrolled leakage from necrotic cells and inflammatory or vascular endothelial cell release.

Adenosine can increase dramatically from the basal level, estimate to be in the range of 30-200 nM. Very minor changes in steady state ATP levels in the cell will translate into major changes in the intracellular adenosine concentrations (100,000 times higher). The adenosine production from hydrolysis of adenosine nucleotides, from ATP or ADP breakdown is controlled through a two-step enzymatic reaction: conversion of ATP or

ADP to AMP by ENTPD1 or CD39 followed by AMP hydrolysis to adenosine by ecto-5'-nucleotidase (NT5E or CD53) [16]. Adenosine generated intracellulary is transported into the extracellular space mainly via specific bi-directional transporters through facilitated diffusion. In some tissues there are nucleoside transporter protein capable of maintaining high adenosine concentration gradient: ENT1 and ENT2, CTN1 and CTN2 [17,18].

Adenosine is formed intracellulary whenever there is a discrepancy between the rates of ATP synthesis and ATP utilization, for example when work load is markedly enhanced or when the supply of oxygen and glucose is limiting as in ischemia. In ischemic areas of after a massive tissue trauma leading to cell death by necrosis can increase to perhaps 30 μM [19].

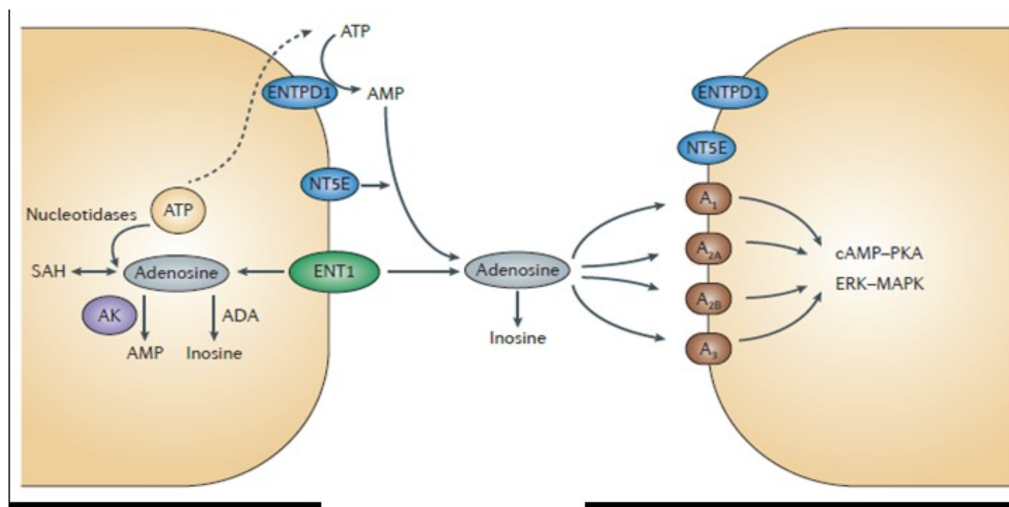


Fig.3 Adenosine synthesis, metabolism and transport in physiological condition [7].

The malfunction of adenosine metabolism is the cause of some serious pathologies. In human, absence of adenosine deaminase induces immunodeficiency (SCID-ADA). Patients with this pathology show skeletal [20], lung, liver [21] and neural abnormalities [22]. The absence of ADA induces increase of deoxyadenosine converted in deoxyadenosinetriphosphatase (dATP) by adenosine kinase. dATP causes lymphocytoxicity in thymus through induction of apoptosis in developing thymocytes [23].

ADENOSINE RECEPTORS

Forty years ago was postulated the existence of adenosine receptors, an hypothesis based on the competitive antagonist of adenosine activity by methylxanthines. Twenty years later four receptors were cloned from several mammalian species and identified as members of G protein-coupled family. These receptors show an high sequence homology among different species [24].

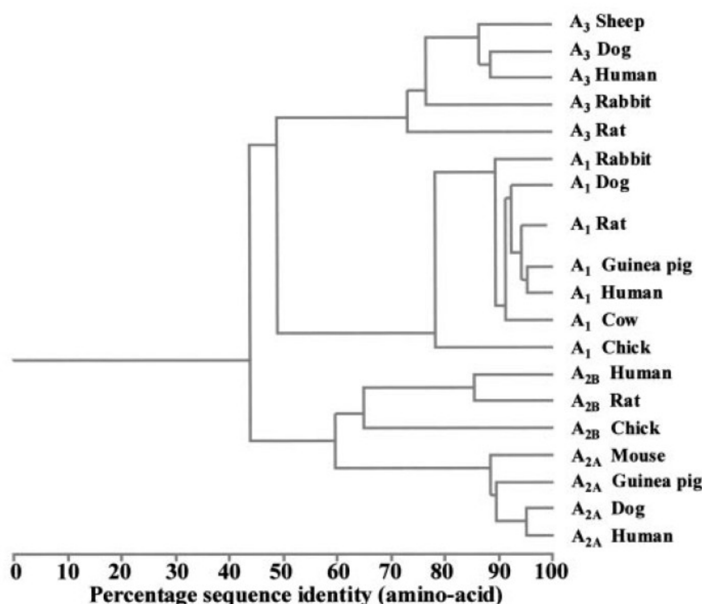


Fig.4 Tree graph showing adenosine receptors gene homology among different species [24].

A_1 adenosine receptor

A_1 adenosine receptor (A_1AR) is the most conserved receptor subtype among species. The gene, ADORA1, have chromosomal localization 1q32.1. It is expressed throughout the body with the highest levels found in the brain (cortex, hippocampus and cerebellum) especially at excitatory nerve endings, but also in heart, kidney, testis and adipose tissue. The A_1AR has been shown to couple with G_i-1, G_i-2, G_i-3 and G_0 but not with G_s or G_z proteins. Responses to A_1ARs activation are blocked by pertussis toxin which is compatible with an involvement of the G_i/G_0 family of G-proteins [14]. The activation of A_1R induces inhibition of adenylate cyclase, activates potassium channels, block transiently calcium and IP_3 levels by activating PLC. A model for this receptor was proposed after studies based on sequence analysis and computer-assisted molecular modeling [25].

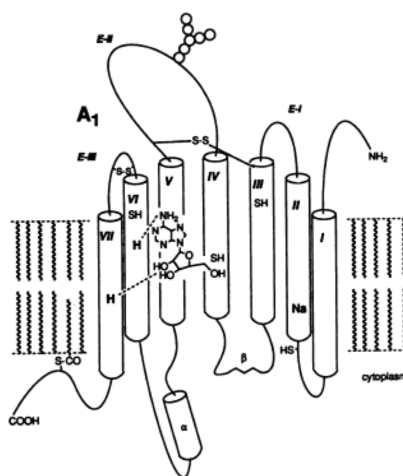


Fig.5 Model of A_1AR protein based on sequence analysis and computer-assisted molecular modeling [14].

Chemical modification of histidine residues in the receptors transmembrane domain, one in helix VI and one in helix VII, strongly affects ligand binding. Glutamate residues in the second extracellular loop as being important for ligand recognition. From extensive mutagenesis analysis, consisting of single aminoacid replacement in human, canine and bovine species, other aminoacids are resulted very important in ligand recognition [24].

Biological functions of A_1AR were studied in mice lacking of ADORA1 gene. This animals showed a reduction of fertility, lifespan [26] and an increase of seizures risk [27]. A_1AR in afferent arterioles mediate vasoconstriction and inhibition of renin secretion and tubular A_1AR appear to modify tubular NaCl absorption [28]. In brain A_1AR are down-regulated by hypoxia in glioma cells and up-regulates in human temporal lobe in epilepsy. A deeply hypothermic and hypometabolic state was pharmacologically induced in a nonhibernating rat by A_1AR agonist CHA [29]. In murine astrocytes A_1AR indirectly reduce the LPS-mediated HIF-1α accumulation, a master regulator of oxygen homeostasis [30]. It is known that astrocytes regulate the reduction of depressive symptoms after sleep deprivation, an effect that seems to be mediate by A_1AR: pharmacological activation of this receptor mimicked the effect of sleep deprivation on depression phenotypes [31]. It has been already demonstrated that IL-6 can increase the survival of retinal ganglion cells in culture and this trophic effect is mediated by adenosine receptor A_1AR activation [32].

Activation of A_1AR in the central and peripheral nervous systems produces an antinociceptive effect. The local application of CCPA in a pain model induced by a

tibial nerve injury inhibited thermal hyperalgesia, but was less effective against mechanical allodynia [33]. The analgesic effect during inflammation could be ascribes at the activation of opioid receptors [34].

Activation of the A_1AR in adipocytes causes inhibition of lipolysis also in pathological conditions like insulin resistance, diabetes and dyslipidemia [35].

From a study that compare WT and A_1AR KO mice results that after administration of an adenosine analogue, insulin induced lipogenesis in A_1AR (+/+), but not in A_1R (-/-) adipocytes. Body weight was similar in young A_1AR (+/+) and A_1AR (-/-) mice, but old male A_1AR (-/-) mice were heavier than wild type controls. Plasma levels of free fatty acids, glycerol and triglycerides were significantly lower in A_1AR (+/+) than in A_1AR (-/-) [36]. After 8-16 h of CPA injection increase of 2-to 10-fold serum level of leptin, the appetite hormone [37].

A_{2A} adenosine receptor

A_{2A} adenosine receptor (A_{2A}AR) gene is localized on 22q11.23 chromosome, have multiple exons, which encode alternative transcripts, whose expression is driven by at least four independent promoters. The regulation of these promoters is highly responsive to alterations in the extracellular environment and particular sensitive to changes in the concentrations of exogenous and endogenous factors involved in inflammation [38]. This is a receptor coupled to Gs protein, and if over-expressed can interact with other G-proteins like G_{olf} in striatum [39].

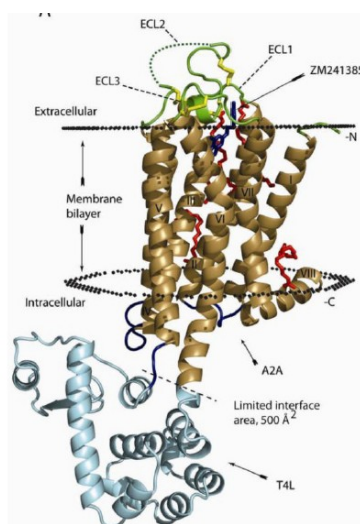


Fig.6 Crystal structure of A_{2A}AR bound to the antagonist ZM 241385 [40].

Stimulation of $A_{2A}AR$ activates PKA that phosphorylates CREB on Ser 133. CREB activation competed with NFkB-p65 for CBP mediating the anti-inflammatory effect [41]. $A_{2A}AR$ have independent G-protein action by C-terminus tail (120 aminoacids highly conserved among species) interaction with other proteins. One of this protein is F-actin, cross linking protein that mediates the internalization of $A_{2A}ARs$ after stimulation contributing to receptor desensitization [42].

High levels of $A_{2A}AR$ are found in the striatum, immune cells of the spleen, thymus, leukocytes and blood plates, but also, any less, in heart, lung and blood vessels [7], where is involved in many physiological and pathological process. $A_{2A}AR$ in the brain interacts with several neurotransmitters to regulate motor activity, psychiatric behaviors, sleep-wake cycle and neuronal cell death. In peripheral tissue $A_{2A}AR$ have a crucial role in the modulation of inflammation, myocardial oxygen consumption, coronary blood flow, angiogenesis and control of cancer pathogenesis [7]. In immune system adenosine binding $A_{2A}AR$ controls lymphocyte trafficking in response to tissue injury or infection mediating extravasations of lymphocytes through blood vessels.

It was demonstrated that activation of $A_{2A}AR$ significantly suppresses the deposition of collagen types I and III, reduces the infiltration of CD4+ T lymphocytes, and attenuates the expression of TGF-β1 and ROCK1, in a mouse model of renal interstitial fibrosis, a pathological process of chronic kidney diseases leading to renal function deterioration [43].

Adenosine regulates the function of the innate and adaptive immune systems mainly through $A_{2A}AR$ activation on monocytes/macrophages, dendritic cells, mast cells, neutrophils, endothelial cells, eosinophils, epithelial cells, as well as lymphocytes, NK cells, and NKT cells [38].

In the heart $A_{2A}AR$ engagement protects against ischemia-reperfusion injury following an acute ischemic episode and inducing profound oxidative/nitrosative stress, capillary pluging, edema and reduction in coronary vascular flow [44]. In the lung inosine acting on $A_{2A}ARs$ can regulate ovalbumin induced allergic inflammation modulating inflammatory processes and cytokines release [45].

In lung, deficiency of $A_{2A}AR$ increases activation of proinflammatory transcription factor Nf-KB and augmented expression of inducible NO synthase [46]. Moreover adenosine by this receptor reduces bowel inflammation [47].

In the liver $A_{2A}AR$ is expressed on Kuppfer cells, hepatocytes and hepatic stellate cells. Engaging of $A_{2A}AR$ induces a reduction of inflammatory cytokines in a model of liver

injury [48] and ischemia reperfusion injury [49]. In the kidney $A_{2A}AR$ is expressed in microvasculature [50] and his activation reduces injury following ischemia-reperfusion in rats [51].

Methotrexate, a therapeutic drug for rheumatoid arthritis, has immunosuppressive and anti-inflammatory effects mediated by increasing of adenosine levels, effect less effective in $A_{2A}AR$ KO mice [52]. In the joint $A_{2A}AR$ exert his anti-inflammatory action in leukocytes, chondrocytes [53] and synovial cells [38]. $A_{2A}ARs$ regulate also the intrinsic circadian clock in immune cells playing an important role in circadian rhythms in rheumatoid arthritis [54]. It was investigated $A_{2A}AR$ density and functionality in RA progression by using a longitudinal study in patients afflicted by rheumatoid arthritis before and after methotrexate, anti-TNFα agents or rituximab treatments: in lymphocytes obtained from RA patients, the $A_{2A}AR$ up-regulation was gradually reduced in function of the treatment time. Moreover in adjuvant-induced arthritis model in rats it was showed the efficacy of the $A_{2A}AR$ agonist, CGS 21680, in comparison with standard therapies [55].

Within brain, $A_{2A}AR$ expression levels are highly concentrated in dorsal and ventral striatum (on striatopallidal) as well as in olfactory tubercle. It is also recognized that $A_{2A}ARs$ are expressed at substantially lower levels outside striatum in brain regions including hippocampus and cortex [56].

$A_{2A}AR$ is involved in sleep induction and it was demonstrate that caffeine induces wakefulness in A_1AR but not in $A_{2A}AR$ KO mice, demonstrating that this effect is mediated by antagonism on $A_{2A}AR$ [57,58]. A genetic variant of the $A_{2A}AR$ gene in humans is associated with individual sensitivity to effect of caffeine on sleep [59].

KO mice for $A_{2A}AR$ show a reduced explorative activity, higher anxiety, slow response to acute pain stimuli and male are much more aggressive than WT mice [60].

$A_{2A}AR$ inactivation in the brain has been associated with protection against brain damage after ischemia, excitotoxicity, traumatic injury and neurodegeneration in Parkinson and Alzheimer disease. Adult mice exposed to $A_{2A}AR$ antagonists including caffeine during pregnancy and lactation displayed loss of hippocampal GABA neurons and some cognitive deficits demonstrating the important role of $A_{2A}AR$ on the neural development [61].

Interestingly, while pharmacological blockade of $A_{2A}AR$ by antagonists induces motor stimulation, adult gb-$A_{2A}AR$ KO mice consistently exhibit reduced spontaneous activity compared to their WT [62], a discrepancy that may relate to partial blockade and/or

acute/short-term blockade of A_{2A}ARs by selective antagonists compared to complete absence and/or long-term depletion of the receptor in these knockout models [63].

The involvement of A_{2A}ARs in neurodegenerative disease, where the inflammatory component is considerable, was deeply investigated by our research group. Data from a study on Huntington's disease patients indicate the existence of an aberrant A_{2A}AR phenotype in the peripheral blood cells of subjects carrying the HD mutation [64] and the alteration of A_{2A}AR in lymphocytes reflects the presence of the mutant protein [65]. In R6/2 HD transgenic mouse model, it was seen a transient increase in A_{2A}AR density and A_{2A}AR cAMP production at early presymptomatic ages and a decreasing A_{2A}AR mRNA, a discrepancy that suggests a compensatory mechanism [66]. A positive correlation was found between A_{2A}AR density in lymphocytes of patients afflicted of neurodegenerative diseases and gravity and progression of the pathology, like as Amyotrophic and Lateral Sclerosis, Multiple Sclerosis and Parkinson's disease [67-69] indicating a possible protective effect of this receptor subtype.

A_{2A}AR play a major role in coronary vasodilatatory properties of adenosine, and it is expressed in coronary endothelial cells as well as in coronary smooth muscle cells. Along with vascular cells, A_{2A}AR is expressed in additional cell types in the myocardium [70]. Many studies show that optimal A_{2A}AR agonist-mediated protection occurs with low doses infused for at least 1 hour. Higher concentrations are associated with significant hypotension and reflex tachycardia and could also induce coronary steal [71,72].

A_{2B} adenosine receptor

A_{2B} adenosine receptor (A_{2B}AR) is codificated by ADORA2B gene with chromosomic localization 17p12-p11.2 and contains a single intron that interrupt the coding region [73].

A_{2B}ARs are expressed on various cells of hematopoietic origin: neutrophils [74], lymphocytes [75] and even platelets [76].

A_{2B}AR is rarely achieved under physiological conditions because requires micromolar adenosine concentrations. Nevertheless Adora2B-KO mice have very strong phenotypes, show low-grade inflammation [77] and vascular leakiness in several organs [78]. During conditions in which adenosine levels are elevated, such as hypoxia, ischaemia or inflammation, A_{2B}AR is involved in tissue adaptation to hypoxia, increased ischemic tolerance or attenuation of acute inflammation [7]. Further studies

identified a strong dependence of $A_{2B}AR$ by HIF-1α: binding of HIF to a hypoxia responsive element on the promoter resulting in increased of $A_{2B}AR$ mRNA and protein [79].

Inflammatory mediators associated with inflammation have been demonstrated to increase $A_{2B}AR$ gene transcription, like bacterial lipopolysaccharide, TNF-α and IL-1β [80]. Other inflammatory mediator including PGE2, IL-6 and IL-4 are not associated with an increase in transcription but rather with stabilization of $A_{2B}AR$ mRNA during inflammation.

$A_{2B}AR$ couples to Gs to activate adenyl cyclase and increase intracellular cAMP levels [81] and can couple to the Gq family of G-proteins to activate PLC and increase intracellular calcium [82]. It appears the G protein coupling of the receptor is cell type dependent and coupling to Gs results in an anti-inflammatory response while coupling to Gq could potentially results in a pro-inflammatory response [80].

Activation of the $A_{2B}AR$ has been shown to promote bone cell differentiation, control glucose homeostasis [83,84] and regulate hyperlipidaemia and atherosclerosis [85]. Stimulation of $A_{2B}AR$ protects against trauma-hemorrhagic shock-induced lung injury [86].

$A_{2B}AR$ promote tumor cell growth by VEGF in Lewis lung carcinoma $A_{2B}KO$ mice: these mice exhibited significantly attenuated tumor growth and longer survival time compared to WT controls [87].

Pharmacologic activation of $A_{2B}AR$ results in proinflammatory effects relevant to the progression of asthma but genetic removal of $A_{2B}ARs$ leads to exaggerate responses in models of acute inflammation. $A_{2B}AR$ gene ablation in ovalbumin-induced chronic pulmonary inflammation attenuates allergen-induced chronic pulmonary inflammation [88].

In a mouse model of COPD it was demonstrated that the blockade of ADORA2B is able to attenuate the development of a pulmonary hypertension phenotype that correlates with reduced levels of hyaluronan deposition in the vessels and down regulation of genes involved in the synthesis of hyaluronan [89].

Conversely, $A_{2B}ARs$ were proposed to play an inhibitory role in degranulation of mouse bone marrow derived mast cells (BMMCs), based on the finding that $A_{2B}KO$ mice show an exaggerated antigen-induced mast cell degranulation [90]. Studies in HMC-1 cells showed that only $A_{2B}AR$, but not $A_{2A}ARs$ or A_3ARs stimulate secretion of angiogenic factors IL-8 and VEGF, and the Th2 cytokines IL-13 and IL-4 [91-94].

A_{2B}ARs expression has been detected in vascular endothelium and smooth muscle cells where it has been implicated in the regulation of vascular tone through receptor-mediated vaso-dilatory effects counteracting A_1AR-mediated vasoconstriction [95].
Due to their large surface area, mucosal organs are particularly prone to hypoxia and ischemia associated inflammation then adenosine via the A_{2B}ARs dampens mucosal inflammation and tissue injury during intestinal ischemia or colitis [96], and A_{2B}AR KO mice show a reduction in induced colonic inflammation compared with their wild-type counterparts [97].

A_3 adenosine receptor

The human A_3 adenosine receptor (A_3AR) was cloned in 1993 from a striatal cDNA library. There is a considerable variation in the pharmacology and distribution, and hence function, of A_3ARs among species. The protein, of 318 amino acids, exhibits almost 30% difference at the amino acid level between human and rat [7].
KO of the A_3AR in mice was surprising, resulting in marked phenotypes even at locations where the receptors are very sparse.
The phenotype of mice that lack the A_3AR shows a marked diurnal variation in activity, heart rate and reduction of body temperature [98].
A_3AR represents a good therapeutic target for tumoral pathologies because is overexpressed in cancer and in inflammatory cells, while low expression is found in normal cells. An overexpression was found in different neoplastic cells including leukemia, lymphoma, astrocytoma, melanoma, mesothelioma and pineal tumor cells [99].
Interesting study were made about the increase expression of A_3AR in tumor tissues derived from patients with colon, breast, small cell lung, pancreatic and hepatocellular carcinomas and melanoma in comparison with adjacent normal cells [100]. In breast cancer tissue A_3AR mRNA increase of 1.27-fold respect to normal tissues [101], and in patients with colorectal adenocarcinomas A_3AR expression was higher in cancer tissue compared with normal mucosa from the same individuals, moreover the expression of these receptors in peripheral blood cells was approximately 3-fold higher compared with healthy subjects [102].
The pharmacological stimulation of A_3ARs with agonist IB-MECA resulted in a significant cell growth inhibition of the human cancer cell line MCF [103] and in the

inhibition of androgen-independent PC-3 prostate human carcinoma cells by deregulation of Wnt and NF-kappa B signaling pathways [104].

A_3AR activation with the specific agonist CF101 inhibits the development of cancer growth in a murine model of colon carcinoma via modulation of GSK-3 β and NF-kB [105].

An A_3AR over expression was described in human and experimental animal model of inflammatory disease. A_3ARs reduce the LPS-mediated HIF-1α accumulation in murine astrocytes, resulting in a downregulation of genes involved in inflammation and hypoxic injury [30]. In a murine model of ischemia-reperfusion activation of A_3AR by Cl-IB-MECA attenuates lung dysfunction, inflammation, and neutrophil activation and chemotaxis [106]. In some case disrupting neutrophils function could adversely affect innate immune responses conferring to A_3AR a pro-inflammatory role [107]. In a mice model of acute pancreatitis, administration of IB-MECA attenuate the histological parameters of inflammation [108]. In patients with rheumatoid arthritis, lymphocytes show an increase of A_3ARs in comparison with healthy controls and activation of these receptors inhibit NF-kB pathway and diminished inflammatory cytokines such as TNF-α, IL-1β and IL-6. Moreover A_3AR density inversely correlated with clinical parameters, DAS28 and DAS, suggesting a direct role of the endogenous activation of these receptors in the control of RA joint inflammation [109]. In an experimental model of autoimmune uveitis, CF101 inhibiting pro-inflammatoy cytokines production and apoptosis of inflammatory cells, improved uveitis clinical score [110].

A_3AR could be considered a biological marker because levels of A_3AR in PBMCs mirror the receptor expression levels in tumor or inflammatory tissue: infact, it is known that pro-inflammatory cytokines, especially TNF-α, binding their receptors, initiate downstream signaling that result in an upregulation of transcription factors, like NF-kB, that bind A_3AR promoter region [99]. In vitro and in vivo model of hepatocellular carcinoma, CF102 treatment decreases the expression level of NF-kB, and TNF-α and prevented apoptosis in the liver with a decreased expression levels of pro-apoptotic proteins Bax and Bad. In addition, CF102-induced apoptosis of Hep-3B cells via de-regulation of the PI3K-NF-κB signaling pathway, resulting in up-regulation of pro-apoptotic proteins [111]. Normally, adenosine modulates colonic cholinergic motility via activation of A_3ARs in the myenteric plexus. A_3AR-mediated tonic inhibitory control by adenosine was impaired in inflamed bowel, despite increased density of functioning and pharmacologically recruitable A_3ARs [112].

3. FUTURE: AGONISTS AND/OR ANTAGONISTS OF ADENOSINE RECEPTORS IN CLINICAL PRACTICE

Due to the involment of adenosine in numerous pathological processes (Fig. 7), the importance of its therapeutically targeting is clear. In the beginning, because the complexity of their signaling, adenosine agonists or antagonists cannot be delivered in a manner that is clinically effective and safe [7]. Actually adenosine molecule named Adenocard was approved by Food and Drug Administration (FDA) in 1989 for the treatment of paroxysmal supraventricular tachycardia restoring normal sinus rhythm in patients. Adenoscan was approved by FDA in 1995, indicated as an adjunct to thallium-201 myocardial perfusion scintigraphy in patients unable to exercise adequately for its vasodilatory effect (FDA web site).

In 2008 Regadenoson (Lexiscan, Astellas Pharma), an $A_{2A}AR$ agonist is approved by FDA for myocardial perfusion imaging in patients with suspected coronary disease.

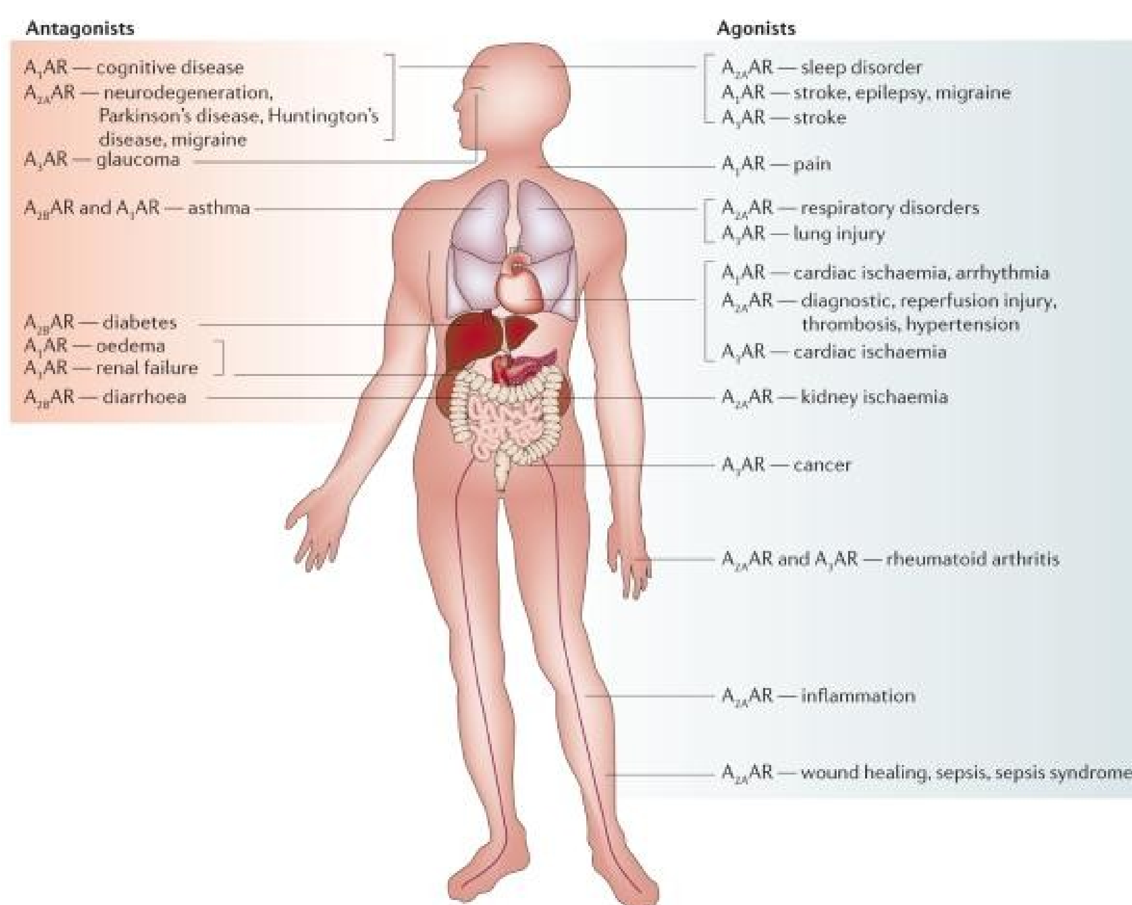


Fig. 7- Possible disease targets for selective adenosine receptor ligands [113].

4. PARKINSON DISEASE

Parkinson's disease (PD) is a pathology with a complex etiology, involving both genetic and environmental factors.

Currently its definition is not so different from the first description made by James Parkinson:

> "Involuntary tremulous motion, with lessened
> muscular power, in parts not in action and even
> when supported; with a propensity to bend the
> trunk forwards, and to pass from a walking to a
> running pace: the senses and intellects
> being uninjured" (J.Parkinson, 1817).

The cardinal signs of PD relate to motor dysfunction and include resting tremor, bradykinesia, rigidity and postural reflex impairment. Other manifestations include psychiatric symptoms such as anxiety and depression and dysautonomic symptoms such as hypotension and constipation, paresthesias, cramps, olfactory dysfunction, seborrheic dermatitis and increase of cognitive deficit with the disease progression.

The main pathological characteristic associated with the motor deficits of PD is degeneration of the dopaminergic neurons of the *pars compacta* of the *substantia nigra* (SNpc) leading to loss of dopamine in the striatum. The loss of these neurons, which normally contains neuromelanin, produces the classic SNpc depigmentation [114]. Symptoms do not develop until about 50–60% of the nigral neurons are lost and 80–85% of the dopamine content of the striatum is depleted.

The combination of asymmetry of symptoms and signs, the presence of a resting tremor with the classic 4-to-6-Hz frequency, and a good response to levodopa best differentiate PD from parkinsonism due to other causes.

PD is characterized by the progressive death of selected but heterogeneous populations of neurons, including the neuromelanin-laden dopaminergic neurons of SNpc, selected dopaminergic brain-stem nuclei (both catecholaminergic and serotoninergic), the cholinergic nucleus basalis of Meynert, hypothalamic neurons, and small cortical neurons (particularly in the cingulate gyrus and entorhinal cortex), as well as the olfactory bulb, sympathetic ganglia, and parasympathetic neurons in the gut [115].

The Lewy Body (LB) is an eosinophilic hyaline inclusion consistently observed in selectively vulnerable neuronal populations. LBs in the brain stem and basal forebrain are usually more than 15 µm in diameter, with a spherical, dense hyaline core, a clear halo.

The LB contains a variety of other constituents, elements of LB filaments, proteins that represent a cellular response to LB formation, enzymes such as phosphatases and kinases, and other cytosolic proteins that probably become trapped in LB during their formation [116]. Elevation of iron levels detected in the pars compacta of the substantia nigra in patients with PD is believed to be an important factor in causing oxidative stress [117]. The metabolism of endogenous dopamine may also produce a number of toxic bioproducts that could contribute to the heightened state of oxidative stress in PD [118].

PD is a complex network disorder in which abnormal activity in groups of neurons in the basal ganglia strongly affect the excitability, oscillatory activity, synchrony and sensory responses of areas of the cerebral cortex that are involved in the planning and execution of movements.

In the basal ganglia function the stimulation of the direct and indirect striatal pathways provokes motor activation and motor inhibition. In PD the equilibrium of the basal ganglia circuits is last due to the depletion in striatal dopamine caused by degeneration of SN dopaminergic neurons [119]. Direct pathway is a monosynaptic inhibitory pathway that is mediated by GABA, whereas the indirect pathway is a polysynaptic net excitatory pathway that includes the *Globus pallidum* (GP) and STN. Input to the striatal neurons comes from cortex and thalamus. Increased activity of the direct pathway results in increased movement via an inhibitory effect on thalamocortical projection neurons, whereas increased activity of the indirect pathway results in the opposite effect. Dopamine receptors in the motor circuit can be either excitatory (D_1) or inhibitory (D_2). The release of striatal dopamine decrease GPi and SNr activity via stimulation of the direct pathway (D_1 receptors in the SNc) and inhibition of the indirect pathway (D_2 receptors). Inhibition of dopamine release has the opposite effect [120].

DA deficiency causes overactivity of the indirect pathway, resulting in an excessive glutamatergic stimulation drive to the internal segment of the GP and pars reticulata of the SN and reduced activity of the inhibitory GABAergic direct pathway, further disinhibiting the activity of the internal segment of the GP and of the SNr. Because these structures use the inhibitory neurotransmitter GABA, the increased output of the

basal ganglia leads to excessive inhibition and, effectively, to a shutdown of the thalamic and brainstem nuclei that receive their outflow (Fig.8). The excessive thalamic inhibition leads to suppression of the cortical motor system, possibly resulting in akinesia, rigidity, and tremor, whereas the inhibitory descending projection to brain-stem locomotor areas may contribute to abnormalities of gait and posture [115].

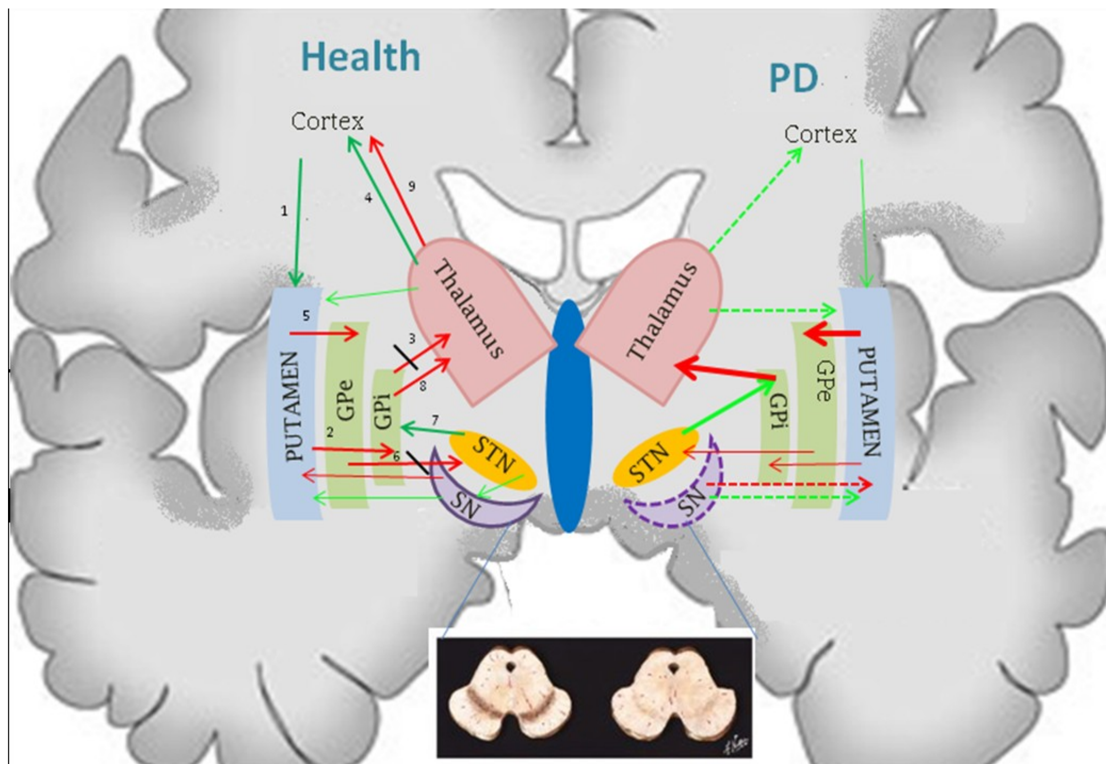


Fig. 8 - Basal ganglia neurotransmission in health and Parkinson's Disease. Numbers (from 1 to 9) indicate the neuro-stimulation/inhibition motor control pathway (red arrow: inhibition of neurotransmission; green arrow: stimulation of neurotransmission; dotted arrow: impairment of the neurotransmission).

EPIDEMIOLOGICAL INFORMATIONS

PD is a very common disease with a decline in incidence in older age groups which may be the result of difficulties in assigning a PD diagnosis in patients with extensive co-morbidities.

Incidence rates for PD in studies that reported results for all age groups ranged between 1.5 and 22 per 100,000 person-years; studies restricted to older populations (above 55 or 65 years) reported overall incidence rates between 410 and 529 per 100,000 person-years. Variations across ethnic groups occur maybe because different environmental exposures and different susceptibility genes in own genetic pattern. In Europe incidence rates is between 9 and 22 per 100,000 person-years, with a rate of 410 and 529 per 100,000 person-years in studies based on populations above 55 or 65 years. In North American studies incidence rates ranged between 11 and 13 per 100,000 person-years, with an incidence rate of 224 per 100,000 person-years in individuals 65 years or above. In Asia incidence rates is between 1.5 and 17 per 100,000 person-years.

The incidence of PD seems to be higher in men than in women whit a ratio of 1.49 and this suggested possible protective effect of estrogens, higher frequency of intensity of occupational toxin exposure and recessive susceptibility genes on the X chromosome. Gender difference decreased with increasing age, as men had higher mortality rates than women.

Overall prevalence ranged between 31 and 970 per 100,000. In US and Europe PD prevalence among people 65 years or older it was estimated at 950 per 100,000. Another study estimated the number of individuals above age 50 with PD in the world at between 4.1 and 4.6 million in 2005. By year 2030 the number was projected to more than double to between 8.7 and 9.3 million [121].

RISK FACTORS

As with many neurodegenerative diseases, age appears to be a clear risk factor for PD: both men and women have an increasing risk of PD with increasing age.

Men have a higher incidence rate of PD than women and one interpretation is that there are potentially protective effects of estrogen because there is some evidence that women with PD were more likely to have undergone a hysterectomy or oophorectomy than those who did not, develop PD. Testosterone could have harmful effects on the development of PD, could be a recessive susceptibility gene on chromosome X, or male lifestyle could predispose to this pathology [122].

Genetic mutations in the α-Syn gene [123] (SNCA) are rare and include point mutations and whole-locus multiplications. Duplications are detected in approximately 1% to 2% of the PD families, and these patients often display a classical PD phenotype; triplications and point mutations are exceedingly rare, found in a few families of Greek, German and Spanish origin and show more severe phenotypes [124,125].

Mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common known cause of autosomal dominant PD. LRRK2 mutations explain approximately 10% of patients and the most common mutation is Gly2019Ser. This mutation is frequent in some populations from South Europe, but very common among Arab patients from North Africa and among Ashkenazi Jewish patients [126].

The most recently described gene involved in autosomal dominant PD is VPS35 within affected members of a Swiss kindred with late-onset, autosomal dominant PD.

Mutation in PRKN, PINK1, and DJ-1 genes cause autosomal-recessive forms of PD [127,128]. Mutations in ATP13A2 (PARK9), PLA2G6 (PARK14), and FBXO7 (PARK15), cause more-rare forms of recessive parkinsonism, usually with very early onset (<30 years) and atypical clinical features [129].

PD is associated with rural living or farming and numerous epidemiological studies have suggested that pesticide or herbicides exposure is closely related to the development of PD, with a risk increased of 1.8-fold (pesticide 1.6-fold and to herbicides 1.4-fold) [130]. No association was observed with fungicides, rodenticides, organochlorines, and organophosphates but 2-fold increase in risk for exposure to paraquat [131].

MPTP, a meperidine analogue occasionally used accidentally by heroin addicts, is a potent neurotoxin with selective effects on nigral dopaminergic neurons. MPTP toxicity is due to the inhibition of complex I (NADH–ubiquinone oxidoreductase) of the mitochondrial electron-transport chain, leading to energy failure and cell death.

INFLAMMATORY COMPONENT IN PD

The central nervous system was supposed to be an “immune privileged” site, in which immune cells of the periphery could not enter or rarely entered, and thus the two systems had little to no interaction.

Today we know that peripheral immune responses can trigger inflammation and exacerbation of CNS degeneration in several neurodegenerative diseases, increasing

neuronal vulnerability to other neurodegenerative insults, such as those caused by aging, oxidative stress, environmental toxins or genetic predisposition (fig.9) [132].

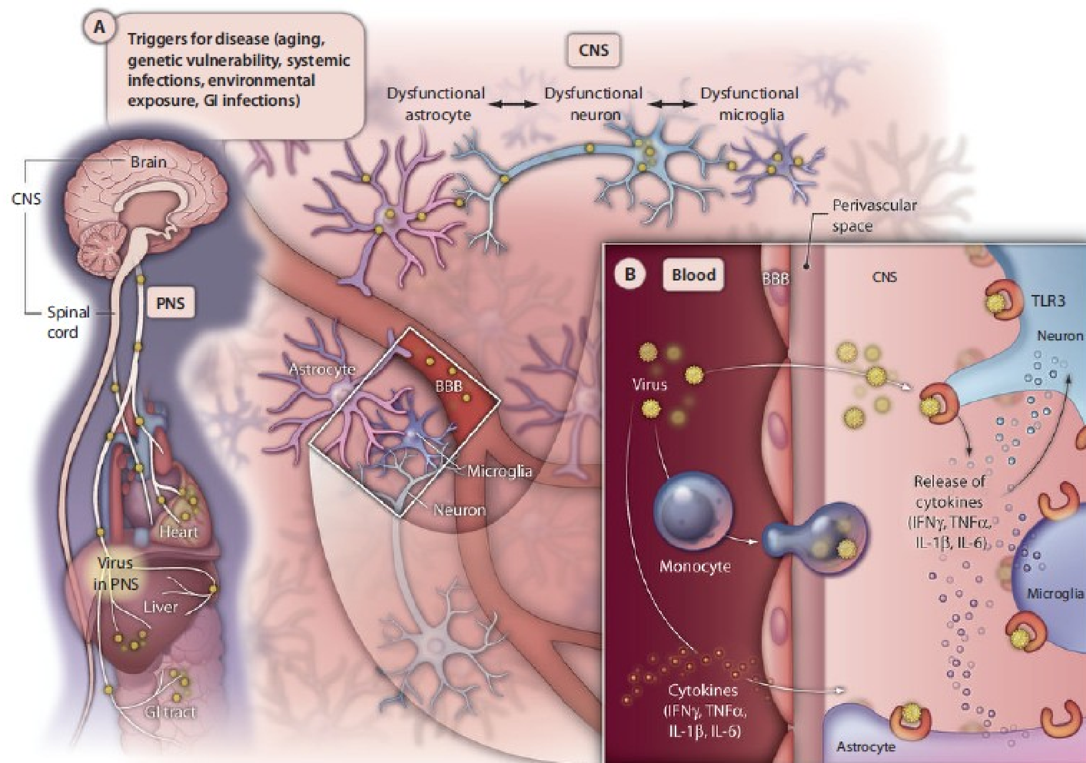


Fig.9- Peripheral inflammatory reactions can also initiate neurodegenerative changes that are often associated with the preclinical phase of many neurodegenerative disease [132].

The cause of the neuronal loss in PD is poorly understood but neuroinflammatory mechanism might contribute to the cascade of events leading to neuronal degeneration. This mechanism comprise microglial activation, astrogliosis, and lymphocytic infiltration. Inflammation in PD is not merely a consequence of neuronal degeneration but may be involved in its progression by producing deleterious proinflammatory molecules [133]. Data from post-mortem studies revealed the presence of activated microglia cells within the substantia nigra of PD patients [134]. The density of astrocytes in SNpc is lower in post mortem brain patients than in healthy individuals, this implies few surrounding cells witch detoxify oxygen free radicals [135]. In addition some peripheral cells can enter the brain parenchyma during the neurodegenerative process because changes in blood brain barrier function might occur in the brain of patients with PD, in fact was found an increase density of endothelial cells and change in brain capillaries in the SN of patients was found, maybe like a consequence of reactive species released by activate microglia [136]. In striatum of post mortem brain

of patients it was found an increase of TNF-α, TGF-β1, IL-1β, IL-6 and IL-2. Furthermore dopaminergic neurons express receptors for these cytokines suggesting that they are sensitive to them which in this way can exert toxic effects [133]. Proinflammatory cytokines can indirectly induce a toxic effect leading expression of the inducible form of NOS or COX- 2 [137]. In body fluids of PD patients there is an increase of active T-cells, CD4+ bright CD8+ dull lymphocytes usually increase after a viral infection, a condition that might contribute to the pathogenesis of the disease [138]. Other studies, supporting the inflammatory role in PD progression, show that men with high levels of IL-6 in blood have increased risk to develop PD. However the risk of this disorder was lower in people who regularly took non-steroidal anti-inflammatory drugs [139].

A recent study provides evidence that enteric inflammation, occurring in PD patients, reinforce the role of peripheral inflammation in the initiation and/or the progression of the disease [140].

LRRK2 is a gene related to Parkinson's disease susceptibility and also to Crohn's disease and numerous scientist support the hypothesis that the detrimental effects mediated by Parkinson's disease LRRK2 mutations may initiate in the periphery and extend to the central nervous system as a consequence of increased levels of pro-inflammatory factors permeable to the blood brain barrier [141].

MANAGEMENT OF PD

The management of PD includes symptomatic and regenerative treatments. Because of the link to dopamine depletion, treatment of PD has centered on various dopamine replacement strategies including dopamine precursor, dopamine agonist and agents that inhibit the metabolism of dopamine.

MAO-B inhibitors were developed as a means to increase synaptic dopamine levels and half-life. Two MAO-B inhibitors, selegilin and rasagiline, are currently licensed in Europe and North America for the symptomatic improvement of early Parkinson's disease. A third MAO-B inhibitor (safinamide) is currently under development in phase III clinical trials as adjuvant therapy to either a dopamine agonist or levodopa [142].

Levodopa/Carbidopa, respectively, Levodopa/Benserazide are the most effective treatment for PD. Levodopa induces complications such as dyskinesias motor fluctuations: "wearing-off " and "on–off " phenomena.

Motor fluctuations occur in 50 percent of patients after 5 years of levodopa therapy and the proportion affected increases to 70 percent among those treated for more than 15 years. Wearing off can be defined as a perception of loss of mobility or dexterity, usually taking place gradually over a period of minutes (up to an hour) and usually having a close temporal relation to the timing of antiparkinsonian medications. On–off effects are unpredictable and generally sudden occurrences (lasting seconds to minutes) of shifts between on and off periods that are not apparently related to the timing of antiparkinsonian medication. These off periods last minutes to hours and do not include transient episodes of "freezing" (also referred to as "motor blocks"; the initiation or continuation of a motor act such as walking is arrested for few seconds) or stress-induced tremor, which are both components of the underlying disease and occur even in the absence of treatment. Once fluctuations and dyskinesia emerge, the pharmacodynamic response changes, resulting in a narrowing of the "therapeutic window" and a specific levodopa threshold is needed for a sufficient clinical response [143].

Diagnoses

McGhee and colleagues summarized the main characteristics of a good biomarker for PD: his change have to follow the neurodegeneration, have to show an association with the clinical phenotype, have a direct association with the disease progression and have not be influenced by symptomatic treatment, be sensitive reflecting small changes in disease progression, quick and cheap to measure, safe and tolerable for the patients, suitable for measurement across different centers. They analyzed numerous scientific work on serum/plasma/blood biomarkers, brain SPECT, brain PET, CSF, brain MRI, concluding that they found insufficient evidence to recommend the use of any biomarker for disease progression in PD [144].

Diagnoses in PD is based on medical history of patients, evaluation of signs and symptoms, neurological and physical examination. Imaging test includes single proton emission computed tomography (SPECT), sonography, positron emission tomography (PET), functional magnetic resonance imaging (fMRI) [145]. In 2011 the FDA approved a specialized imaging technique called DaTscan that allows doctors to capture detailed pictures of the dopamine system in brain. Unfortunately, because there is no definitive test for PD, and because these disease symptoms are similar to those of other neurological conditions, the misdiagnosis rate remains significant. PET and SPECT

imaging use a number of radiotracers for in vivo assessment of normal and abnormal brain function. Cerebral blood flow and glucose can be mapped with radiolabeled water or glucose. SPECT imaging is less expensive and more available than PET that has a superior spatial resolution and higher sensitivity. fMRI is available, don't require the injection of a radiotracer [146].

The Unified Parkinson's Disease Rating Scale (UPDRS) is a scale that was developed as an effort to incorporate elements from existing scales to provide a comprehensive means to monitor PD, related disability and impairment. The scale has four components, largely derived from preexisting scales: part I-Mentation, Behaviour and Mood; part II-Activities of daily living; Part III-Motor; Part IV-complications. UPDRS is often accompanied with Hoehn and Yahr scale [147]. Hoehn and Yahr scale analyzed the motor decline progression, deterioration in quality of life, neuroimaging studies of dopaminergic loss and it is focused on issues of unilateral versus bilateral disease [148]. The "mini mental state examination" (MMSE) is useful for the evaluation of the cognitive deficit and it is named "mini" because excludes questions concerning mood and abnormal mental experience [149]. Usually for the PD diagnosis are used also the Geriatric Depression Scale (GDS) and the Frontal Assessment Battery: the first test represent a reliable and valid self-rating depression screening scale for elderly populations [150] and the second is a bedside cognitive and behavioral battery to assess frontal lobe functions [151].

Evidences of A_{2A}AR useful in Parkinson's disease management

In recent years much research was focused on identifying non dopaminegic compounds to be used as adjuvants to L-DOPA therapy. Among several new classes of drugs, A_{2A}AR antagonists have emerged as the most promising candidates [119]. From a 30 years study emerged that incidence of PD decline consistently with increased amount of coffee intake, from 10.4 per 10000 person-years in men who drank no coffee to 1.9 per 10000 person years in men who drank at least 28 oz/day: caffeine, an adenosine receptors antagonist, intake is associated with a significantly lower incidence of PD [152].

Patients with PD have altered adenosine A_{2A}AR expression in the basal ganglia, suggesting a pathogenic role for these receptor. A_{2A}AR mRNA levels (+129%) and [^{3}H]-SCH58261 specific binding (+32%) were increased in the putamen of dyskinetic

patients compared to controls. This increase was also significant compared with dyskinetic PD patient (+60% and +24% for mRNA and [^{3}H]-SCH58261 specific binding respectively). These data suggest that A_{2A}AR increase is associated with the development of dyskinesias [153].

The deletion of the A_{2A}AR in mice protects against dopaminergic neuron degeneration induced by a mutant human α-synuclein transgene. The A_{2A}AR KO completely prevents loss of dopamine and dopaminergic neurons suggesting that A_{2A}ARs appear required for neurotoxicity in a mutant α-synuclein model of PD [154].

The A_{2A}AR is largely coexpressed with D_2DR in the striatum where modulates dopaminergic activity and antagonizes D_2DR mediates neurochemical effects by direct A_{2A}AR-D_2DR interaction. The role of this interaction was analyzed by using mice genetically deficient in A_{2A}ARs or D_2DRs or both. In D_2 KO and wild-type mice the A_{2A}AR agonist reduced spontaneous and amphetamine-induced locomotion, instead in the same mice A_{2A}AR antagonist produce motor stimulation [155].

Neurochemical studies have shown that activation of A_{2A}ARs reduces the binding affinity of D_2 agonist to their receptors [156].

By immunofluorescence experiments was demonstrated the co-localization of A_{2A}AR and D_2DR in cell membranes of SH-SY5Y human neuroblastoma cell stably transfected with human D_2DR and in cultured striatal cells (figure 10). Furthermore long term exposure to A_{2A}AR and D_2DR agonists in D_2DR cotransfected neuroblastoma cells resulted in coaggregation, cointernalization and codesensitization of A_{2A}AR and D_2DR [157].

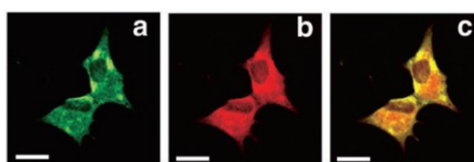


Fig.10- Double immunofluorescence staining in SH-SY5Y neuroblastoma cells stably transfected with the human D_2DR. a) A_{2A}AR immunoreactivity (green); b) D_2DR immunoreactivity (red); c) superimposition of images revealing the colocalization of A_{2A}AR and D_2DR (yellow) [157].

A_{2A}AR and D_2DR have reciprocal antagonistic interactions that regulate GABAergic neurons. A_{2A}AR activation in the indirect pathway opposes the action of D_2DR activation by means of an intramembrane interaction, whereas stimulation of D_2DRs inhibits A_{2A}AR induced activation of adenylyl cyclase. Stimulation of A_{2A}AR facilitates

GABA release whereas D_2DR stimulation inhibits this process. Inhibition of $A_{2A}AR$ and D_2DR produces opposite effects [120].

The antiparkinsonian effect of $A_{2A}AR$ antagonist was well studied in different PD animal models. In 6-OHDA lesioned rats rendered dyskynetic by prior treatment with levodopa, $A_{2A}AR$ antagonist produce and additive reduction in motor disability with levodopa [158]. In MPTP treated common marmosets, oral administration of istradefylline increased locomotor activity [159]. In levodopa primed MPTP treated cynomologous monkeys, istradefylline significantly improved locomotor function, comparable to that observed with levodopa (50 mg) but with little or no dyskinesia [160].

In human a potent and selective $A_{2A}AR$ antagonist, preladenant, was tested on 253 patients of 15 countries. This study revealed that 5 and 10 mg of preladenant twice daily (fig. 11) might be clinically useful to reduce off time in patients with PD and motor fluctuation [161].

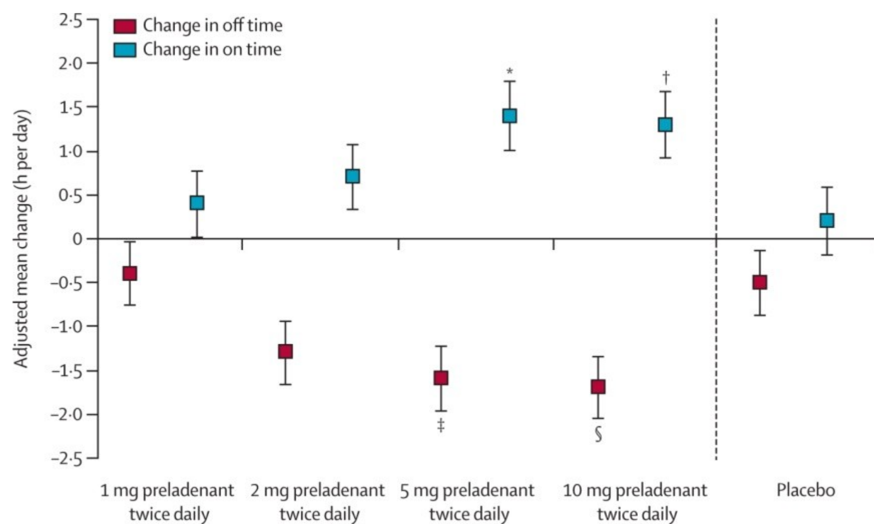


Fig.11- Preladenant administration: mean change in daily off and on time from baseline to week 12. *p=0·024 versus placebo. †p=0·0489 versus placebo. ‡p=0·0486 versus placebo. §p=0·019 versus placebo[161].

CHAPTER II

AIM OF THE STUDY

AIM OF THE STUDY

PD is a neurodegenerative disorder with a strong inflammatory component. Increasing evidences suggest a direct involvement of adenosine in PD and its key role in the regulation of neurotransmission. For example epidemiological studies suggest that higher coffee and caffeine (unspecific antagonist for adenosine receptors) assumption, is associated with a significantly lower incidence of PD [162]. A_{2A} is the main adenosine receptor involved in this disorder. In the brain is localized prevalently in the striatum and it is co-expressed with D_2 dopamine receptors: here exerts an antagonist effect on D_2DR and if up-regulated induces motor impairment.

The aim of this study was to investigate the presence and a possible density and functional alteration of A_{2A}ARs and D_2DRs in post mortem putamen of PD patients compared to control subjects. Furthermore from analysis on blood of patients we found high levels of TNF-α in plasma, then we were interested to understand the involvement of inflammation, in particular of this cytokine, in the neuronal up-regulation of A_{2A}AR. For this purpose we analyzed the effect of TNF-α on A_{2A}AR density and cAMP production in PC12 cell line before and after treatment with NGF to induce a neuronal phenotype in these cells. Moreover we are interested to understand if the involvement of A_{2A}AR in PD is also associated to a modification in dopamine reuptake. For this purpose we measured the dopamine quantity after stimulation and/or inhibition of A_{2A}AR with specific agonist and antagonist.

The other goal of our study is the identification of a possible correlation between A_{2A}AR density in the blood and clinical parameters of PD patients to understand if peripheral A_{2A}AR alteration could mirror the brain damage and then use it like a biomarker for PD. To this end it was evaluated the affinity and density of A_1-, A_{2A}, A_{2B}- and A_3ARs in lymphocyte and neutrophil membranes from PD patients and healthy control subjects. These data were correlated with score values of different scales estimating motor and cognitive impairment of PD patients.

CHAPTER III

METHODS

METHODS

1. Subjects

Brains of postmortem PD patients and healthy controls were derived from Netherlands Brain Bank of the Department of the Netherlands Institute for Neuroscience, Amsterdam, Netherlands. Human brain samples from 7 PD patients (3 men and 4 women) were obtained as well as from 7 controls (3 men and 4 women) who died with no neurological disorders. All autopsies were carried out within 12 hours of death, and the brain fragments were immediately frozen at -80°C until use in the experimental assays (Table 1M).

We collected peripheral blood samples of 129 PD patients (82 men and 47 women) attending the Neurological Clinic of the University of Ferrara, Italy. PD diagnosis was based on established diagnostic criteria. All patients were followed up by the same neurologist. Clinical data were obtained from medical records of patients and direct interviews, throughout a structured questionnaire administered to patients and their relatives. Demographic data, family and clinical history, and age at onset of initial symptoms were recorded. Pharmacological data regarding antiparkinsonian drugs and other concurrent treatments were also registered. The levodopa equivalent daily dose (LEDD) was computed according to standard conversion factors. The severity of PD symptoms and signs was assessed using the Unified Parkinson's Disease Rating Scale (UPDRS) and the Hoehn and Yahr's staging. The mini–mental state examination (MMSE), the Geriatric Depression Scale (GDS), and the Frontal Assessment Battery (FAB) were also administered. The blood samples were obtained from PD patients on the same day of the clinical examination and interview. Clinical features of included PD subjects are summarized in Table 2M.

123 healthy control subjects (79 men and 44 women) were volunteers from Ferrara University Hospital Blood Bank and were matched for similar age to the cohort of PD patients. The study was approved by the local Ethics Committee of the University Hospital of Ferrara, and informed consent was obtained from each participant in accordance with the principles outlined in the Declaration of Helsinki.

NBB autopsy code	Diagnosis	Sex	Age at death (years)	Delay to autopsy (hours:min)	Brain tissue pH
96-032 96/052 191 J	non-demented control	F	60	08:25	6.60
97-043 S97/133 100 W1	non-demented control	M	68	10:10	7.08
93-110 *93/215 119 F	non-demented control	M	73	08:45	6.11
04-049 S04/158 351 B	non-demented control	F	77	08:20	6.48
04-021 S04/057 351 B	non-demented control	M	80	06:30	6.43
98-056 S98/123 100 W1	non-demented control	F	83	05:15	7.30
96-078 S96/238 191 J	non-demented control	F	87	08:00	6.91
03-078 S03/239 300 PUT3	PD with dementia	F	61	04:30	6.30
94-026 S94/069 136 K2	PD	M	68	06:00	6.70
05-080 S05/298 300 PUT3	PD	M	73	06:35	6.28
94-092 S94/245 104 G	PD	F	77	09:40	6.86
93-007 S93/014 104 G	PD	M	79	05:25	6.15
94-021 S94/062 136 K2	PD	F	83	05:00	6.58
05-045 S05/160 300 PUT3	PD with dementia	F	87	05:25	6.44

Table 1M- Characteristics of postmortem healthy controls and PD patients.

Characteristics of PD patients	
Age (mean ± SD)	64.8 (8.4)
Time since diagnosis (median interquartile range) years	5 (3-6)
Hoehn & Yahr staping (mean ± SD)	2.33 (0.8)
UPDRS II (mean ± SD)	9.4 (7.7)
UPDRS III (mean ± SD)	22.8 (14.5)
MMSE score (mean ± SD)	26.2 (3.3)
LEDD (mean ± SD)	546.7 (352)

Table 2M- Demographic and clinical characteristics of included PD patients involved in the blood examinations.

Patients drug assumption (% of patients)	
L-DOPA (monotherapy)	10
DOPAMINE AGONIST (monotherapy)	3.3
POLYTHERAPY	43.3
L-DOPA AND DOPAMINE AGONISTS	36.6
ANTIPSYCHOTICS	6.6
ANTIDEPRESSANTS	33.3

Table 3M- Percentage of PD patients involved in our study that assumed specific drugs.

2. Preparation of human putamen membranes

Human putamen of PD patients and controls was homogenized in 50 mM Tris-HCl buffer, pH 7.4, with a Polytron (Kinematica Inc., Bohemia, NY, USA) and centrifuged for 20 min at 48,000 g. To study A_{2A}ARs, the membrane pellet was suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ and was incubated with 2 IU/ml adenosine deaminase for 30 min at 37°C. Similar aliquots of membranes were suspended in 50 mM Tris-HCl buffer, pH 7.4, with the aim of investigating D_2DRs.

3. Preparation of blood peripheral cells or membranes

Lymphocytes or neutrophils were isolated and prepared from the peripheral blood of PD patients and control subjects. The blood was supplemented with 6% (by weight) Dextran T500 solution (Sigma, St. Louis, MO, USA). Cells were pelletted by centrifugation for 5 min at 250 g, suspended in Krebs-Ringer phosphate buffer, and layered onto 10 ml of Fycoll-Hypaque (GE Healthcare, Little Chalfont, UK). After centrifugation, lymphocytes or neutrophils were diluted at 1 x 10^6 cells/sample and used immediately in cAMP experiments. To obtain membrane suspensions, cell fractions were centrifuged in hypotonic buffer at 20,000 g for 10 min. The pellet was resuspended in Tris-HCl 50 mM buffer, pH 7.4, containing 2 UI/ml adenosine deaminase (Sigma), incubated for 30 min at 37°C, and used for radioligand binding assays. The proteins concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine albumin as the reference standard.

4. PC12 cell culture

PC12 cells were maintained in DMEM medium supplemented with 5% FCS, 10% horse serum, 2 mM glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere (5% CO_2) at 37°C. Cells were subcultured 3x/wk at a density of $5x10^5$/ml. Differentiation was achieved by treatment with 50 ng/ml NGF (Sigma) for 1 week.

Cells were treated with TNF-α (100 ng/ml; Sigma) for 24 h and then harvested for radioligand binding experiments to examine A_{2A}ARs and D_2DRs. PC12 cells were also treated with a potent A_{2A}AR agonist, CGS 21680 (1 µM; Sigma) and with a selective A_{2A}AR antagonist, SCH 58261 (1 µM; Tocris, Bristol, UK) for 24 h and then used in A_{2A}AR and D_2DR saturation binding experiments.

5. Real-time RT-PCR experiments

Total cytoplasmic RNA was extracted from human putamen fractions and human lymphocytes by the acid guanidinium thiocyanate phenol method. Quantitative real-time RT-PCR assay of A_1-, A_{2A}-, A_{2B}-, and A_3R mRNAs was carried out using a gene specific fluorescently labeled TaqMan MGB probe (minor groove binder) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For the real-time RT-PCR of A_1-, A_{2A}-, A_{2B}-, and A_3ARs, the Assay-on-Demand Gene Expression Products NM 000674, NM000675, NM 000676, and NM 000677 (Applied Biosystems) were used, respectively. For the real-time RT-PCR of the reference gene, the endogenous control human β-actin kit was used, and the probe was fluorescently labeled with VIC (Applied Biosystems).

6. Western blotting analysis

Human putamen fractions and human lymphocytes were washed with ice-cold phosphate buffered saline (PBS) containing 1 mM sodium orthovanadate, 10^4 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64. Then cells or tissues were lysed in Triton lysis buffer, and the protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Aliquots of total protein sample (50 µg) were analyzed using antibodies specific for human A_{2A}ARs (Alpha Diagnostic, San Antonio, TX, USA; 1 µg/ml dilution). Filters were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies (1: 2000 dilution).

Specific reactions were revealed with enhanced chemiluminescence Western blotting detection reagent (GE Healthcare).

7. Saturation binding to D_2DRs and ARs

Binding to D_2DRs in human putamen aliquots was determined by using [^{3}H]-spiperone (specific activity 16 Ci/mmol; Perkin Elmer, Boston, MA, USA) as radioligand. The tissue membranes (50 µg protein/assay) were incubated for 15 min at 37°C with 8–10 concentrations of the radioligand [^{3}H]-spiperone (0.05–5 nM). Non specific binding was determined in the presence of 1 µM butaclamole.

Human lymphocyte and neutrophil membranes (60 µg protein/assay) with [^{3}H]-DPCPX (0.01–20 nM) as radioligand (specific activity 120 Ci/mmol; Perkin Elmer) were incubated for 90 min at 25°C. Nonspecific binding was determined in the presence of DPCPX 1 µM. Saturation binding to A_{2A}ARs in human putamen or blood-cell membranes (60 µg protein/assay) was determined by using [^{3}H]-ZM 241385 (0.01–20 nM; specific activity 27 Ci/mmol; Biotrend, Cologne, Germany) as radioligand and were incubated for 60 min at 4°C. Nonspecific binding was determined in the presence of ZM 241385 1 µM. Saturation binding to A_{2B}ARs was determined by incubating cell membranes (80 g protein/assay) and [^{3}H]-MRE 2029F20 (0.01–30 nM) as radioligand (specific activity 123 Ci/mmol; GE Healthcare) for 60 min at 4°C. Nonspecific binding was determined by using MRE2029F20 1 µM. Saturation binding to A_3ARs was determined in membranes (80 µg protein/assay) and [^{3}H]-MRE 3008F20 (0.01–30 nM) as radioligand (specific activity 67 Ci/mmol; GE Healthcare) at 4°C for 150 min. Nonspecific binding was determined in the presence of MRE 3008F20 1 µM. Saturation binding experiments were also carried out in untreated or NGF-treated PC12 cells for A_{2A}ARs and D_2DRs in the absence and in the presence of TNF-α (100 ng/ml), CGS 21680 (1 µM), and SCH 58261 (1 µM). At the end of the incubation time, bound and free radioactivity was separated by filtering the assay mixture through Whatman GF/B glass fiber filters in a Brandel cell harvester (Brandel, Unterföhring, Germany). Filter bound radioactivity was counted in a Packard 2810 TR liquid scintillation counter (Packard Instrument Co., Meriden, CT, USA).

Similar experimental conditions were also carried out in untreated or pharmacologically treated PC12 cells with the aim of verifying the affinity and density of D_2DRs. Experimental condition are summarized in table 4M.

Receptor	Radioligand	Specific bound	[Protein]	Time-Temper.
D_2DR	[^{3}H]-Spiperone 0.05–5 nM	Butaclamole 1 µM	50 µg protein/assay	15 min at 37°C
A_1AR	[^{3}H]-DPCPX 0.01–20 nM	DPCPX 1 µM	60 µg protein/assay	90 min at 25°C
$A_{2A}AR$	[^{3}H]-ZM 241385 0.01–20 nM	ZM 241385 1 µM	60 µg protein/assay	60 min at 4°C
$A_{2B}AR$	[^{3}H]-MRE 2029F20 0.01–30 nM	MRE2029F20 1 µM	60 µg protein/assay	60 min at 4°C
A_3AR	[^{3}H]-MRE 3008F20 0.01–30 nM	MRE 3008F20 1 µM	80 µg protein/assay	150 min at 4°C

Table 4M- Experimental conditions of binding assays for ARs and D_2 dopamine receptors.

8. Measurement of cAMP levels

Human lymphocytes or neutrophils (10^6 cells/sample) were suspended in Krebs Ringer phosphate buffer containing 1.0 IU/ml adenosine deaminase and were preincubated for 10 min in a shaking bath at 37°C. To evaluate the adenylyl cyclase activity and cAMP production, the cells were incubated with forskolin (1 µM), adenylate cyclase activator, and/or Ro 20-1724 (0.5 mM) as phosphodiesterase inhibitor. The effect of typical $A_{2A}AR$ agonists such as CGS21680 and NECA at different concentrations (1 nM-1 µM) was studied. To demonstrate the direct involvement of $A_{2A}ARs$, the effect of a selective $A_{2A}AR$ antagonist (SCH 58261, 1 µM) and a nonselective well-known antagonist (caffeine, 10 µM) was investigated. The final aqueous solution was tested through a competition protein binding assay by using [^{3}H]-cAMP (specific activity 21 Ci/mmol; GE Healthcare), 0.1 M trizma base, 8.0 mM aminophylline, and 6.0 mM mercaptoethanol, pH 7.4. Similar experimental conditions were also used in untreated or NGF-treated PC12 cells.

9. Adenosine concentration in human plasma

Adenosine concentration was evaluated by using a specific sensor that is based on the principle of converting adenosine to inosine, hypoxanthine, and uric acid with the evolution of hydrogen peroxide. The three enzymes, adenosine deaminase, nucleosidase phosphorylase, and xanthine oxidase, required for this process were loaded into single- and dual-barrelled sensor probes obtained from Sycopel International (Jarrow, UK). The

probes were controlled by a potentiostat (Biosensor Driver; Sycopel International) that held the working electrode at +650 mV to detect hydrogen peroxide.

10. TNF-α release in human plasma

TNF-α levels were measured in human plasma obtained from control subjects and PD patients by using a highly sensitive human TNF-α enzyme linked immunosorbent assay (R&D Systems, Minneapolis, MI, USA) according to the manufacturer's instructions.

11. [^{3}H]-dopamine uptake assay

PC12 cells (10^6 cells/ml) were suspended in a modified Krebs HEPES buffer containing 125 mM NaCl, 4.5 mM KCl, 2.2 mM $CaCl_2$, 1.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, 5.6 mM glucose, 100 µM ascorbic acid, 100 µM pargyline, and 100 nM desipramine at pH 7.4. PC12 cells were incubated for 30 min at 37°C with A_{2A}AR agonists such as CGS 21680 and NECA or the A_{2A}AR antagonist SCH 58261 and the nonselective adenosine antagonist caffeine to evaluate the involvement of A_{2A}ARs in dopamine uptake. [^{3}H]-dopamine (50 nM, specific activity 20.5 Ci/mmol; Perkin Elmer) was added to each assay tube, and the cell suspension was incubated for 5 min at 37°C. Nonspecific uptake was defined in the presence of the specific dopamine uptake inhibitor GBR 12783 (10 µM, Tocris). Uptake experiments were stopped by the addition of ice-cold Krebs HEPES buffer and rapid filtration with a Brandel cell harvester through Whatman GF/B glass fiber filters. The filter-bound radioactivity was counted by using a Packard 2500 TR liquid scintillation counter.

12. Data and statistical analysis

Dissociation equilibrium constants for saturation binding, affinity, or K_D values, as well as the maximum densities of specific binding sites (Bmax), were calculated for a system of 1-or 2-binding site populations by nonlinear curve fitting using the program Ligand (Kell Biosoft, Ferguson, MO, USA). Functional experiments were calculated by nonlinear regression analysis using the equation for a sigmoid concentration-response curve (GraphPad Prism; GraphPad, San Diego, CA, USA). Analysis of data was performed by unpaired t test and differences between the groups were considered significant at a value of $p<0.01$. All experimental data are reported as means ± standard error of independent experiments.

Differences between groups were analyzed with Dunnett's test and were considered significant at a value of $p<0.01$.

A simple regression model was used to analyze the linear dependence of clinical variables on Bmax and on the potency (EC_{50}) of typical $A_{2A}AR$ agonists. In addition, correlation between pairs of variables was assessed by means of Spearman's.

Correlation between continuous variables was verified using Person's correlation coefficient and Spearman's correlation coefficient and Spearman's nonparametric correlation coefficient. Data were considered statistically significant at $p <0.01$.

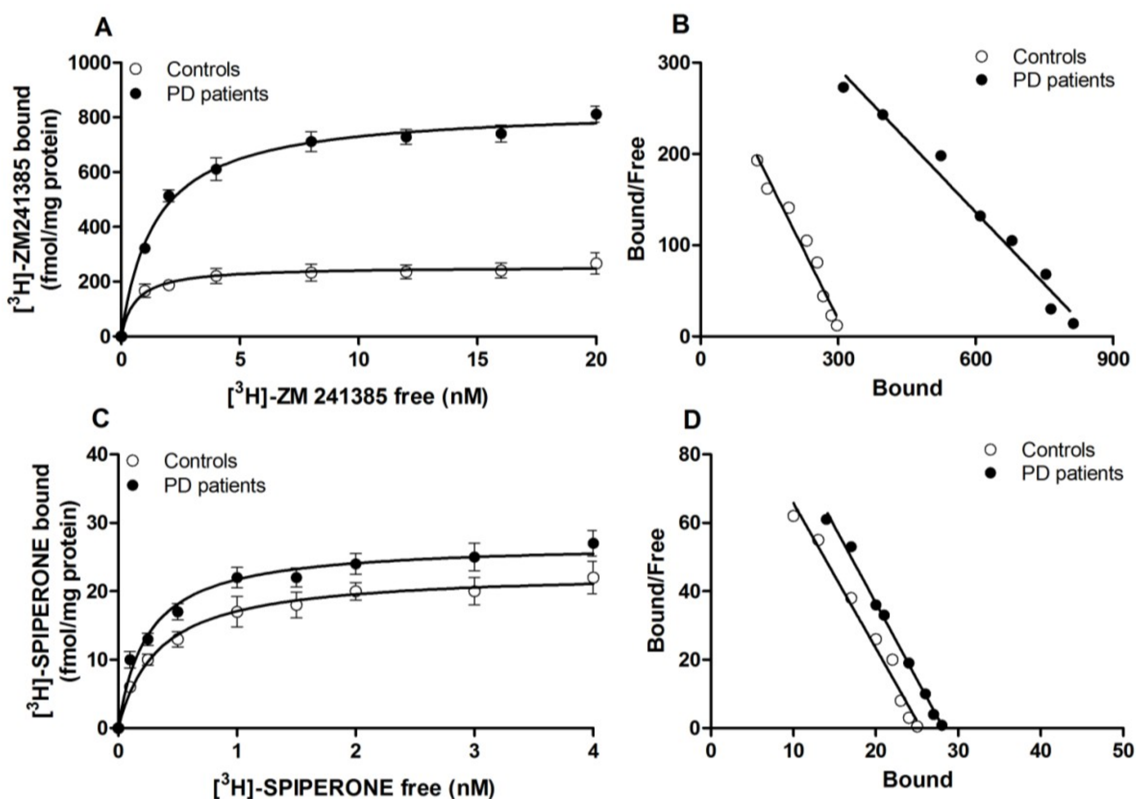
Variables that are significantly related to Bmax and K_D were used as dependent variables in multiple regression analyses. To evaluate the relationship between binary variables (presence/absence of dyskinesias or motor fluctuation) and binding parameters we conducted multivariate logistic regression analyses. Bmax and K_D were categorized by quartiles, the first quartile being used as a reference category. The estimated odds ratios (OR) with 95% confidence interval (CI) were adjusted for age, disease duration and the levodopa equivalent daily dose.

CHAPTER IV
RESULTS

RESULTS

1. A_{2A}Rs and D_2DRs in human putamen

Figure 1R shows A_{2A}AR and D_2DR binding characteristics (affinity and density) in human putamen from 7 control and 7 PD postmortem subjects. [^{3}H]-ZM 241385 saturation curves and Scatchard plots show a statistically significant increase of A_{2A}AR density in PD subjects of 2.95-fold when compared with controls (Bmax=279±33 and 822±89 fmol/mg protein respectively; Fig.1R A, B). D_2DRs (affinity and density Fig. 1R C, D) were found in postmortem control subjects and PD patients, revealing the presence of a single class of high-affinity D_2DRs (K_D=0.30±0.004 nM and 0.37±0.06 nm for control and PD patients respectively) with a low level of expression (Bmax=24±3 and 28±4 fmol/mg protein respectively).



	A_{2A}AR		D_2DR	
	K_D (nM)	B_{max} (fmol/mg protein)	KD (nM)	Bmax (fmol/mg protein)
Control's putamen	0.84±0.07	279±33	0.30±0.04	24±3
PD patients' putamen	1.23±0.28*	822±89*	0.37±0.06	28±4

Fig. 1R- Saturation curves and Scatchard plot on human putamen membranes from postmortem PD and control subjects of A, B) [^{3}H]-ZM 241385 binding to A_{2A}AR and of C, D) [^{3}H]-spiperone binding to D_2DR. Data are expressed as mean ± SE of 7 PD and 7 control subjects. T-test, *$p<0.01$ vs. control group.

2. Evaluation of AR mRNA expression and protein level in putamen

$A_{2A}AR$ mRNA in putamen obtained from control subjects and PD postmortem subjects was assayed by using real-time quantitative RT-PCR (Fig. 2R A). An elevated $A_{2A}AR$ mRNA increase of 5.2-fold was found in the putamen of PD patients in comparison with control subjects (*n*=7). The immunoblot signals of $A_{2A}ARs$ in human putamen aliquots of controls and PD patients suggest that $A_{2A}ARs$ are differentially expressed in healthy humans and in humans with PD (Fig. 2R B). $A_{2A}ARs$ proteins are overexpressed in PD putamen (280%). These data suggest that the elevated expression of $A_{2A}AR$ protein in PD patients is directly associated with an increase in $A_{2A}AR$ mRNA.

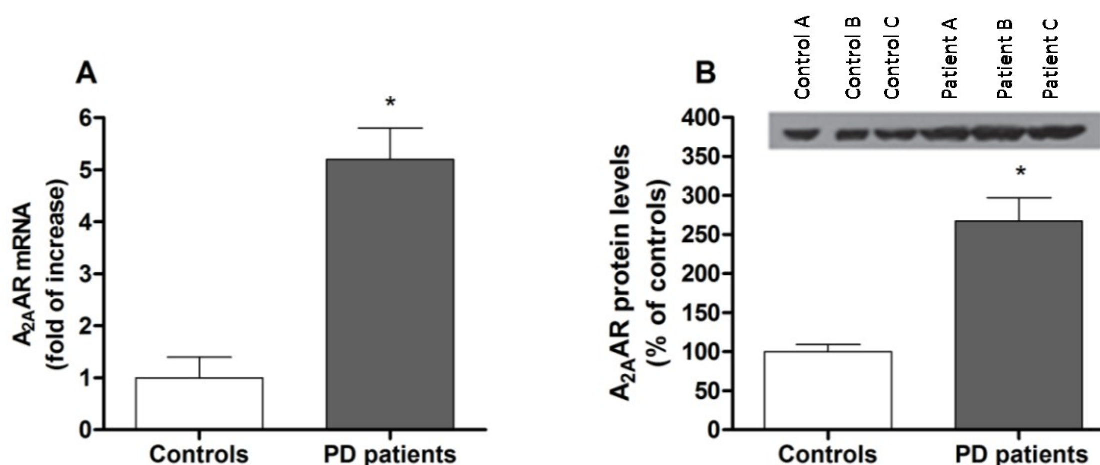


Fig. 2R- $A_{2A}AR$ mRNA (A) and protein (B) levels in putamen tissues of control and PD postmortem subjects. Data are expressed as means± SE. T-test, $p<0.01$ *vs.* control group.

3. Affinity and density of A_1-, A_{2A}-, A_{2B}-, and A_3ARs in peripheral blood cells

Affinity and density of ARs in lymphocyte or neutrophil membranes from the control group (n=83) and PD group (n=89) were evaluated. ARs density showed that only $A_{2A}ARs$ but not A_1-, A_{2B}-, and A_3ARs were significantly different in PD patients when compared with control subjects (Fig. 3R, 4R; Table 2R, 3R). In lymphocyte membranes, the affinity of $A_{2A}ARs$ was significantly decreased ($p<0.01$) in PD patients compared with control subjects. The density of $A_{2A}ARs$ was significantly increased (3.29-fold of increase, $p<0.01$) in the PD group in comparison with the control group (Fig. 4R A, B). Similar results were obtained in neutrophil membranes (2.25-fold of increase, $p<0.01$) confirming an alteration of $A_{2A}ARs$ in PD patients (Fig. 4R C, D).

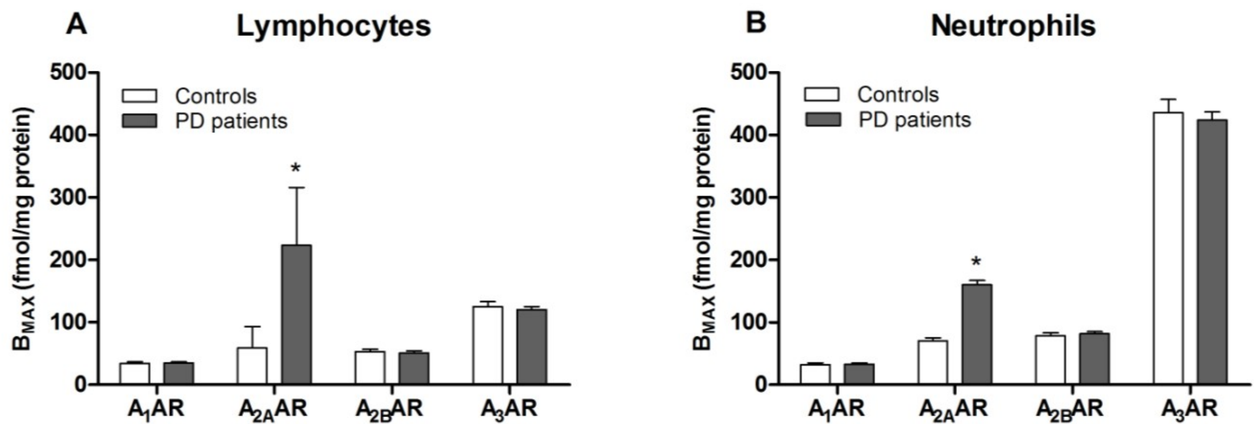


Fig. 3R - Receptor density of A_1-, A_{2A}-, A_{2B}-, and A_3ARs in lymphocytes (A) and neutrophils (B) from PD patients in comparison with healthy control subjects. Data are expressed as means ± SE. T-test, *p < 0.01 *vs.* control group.

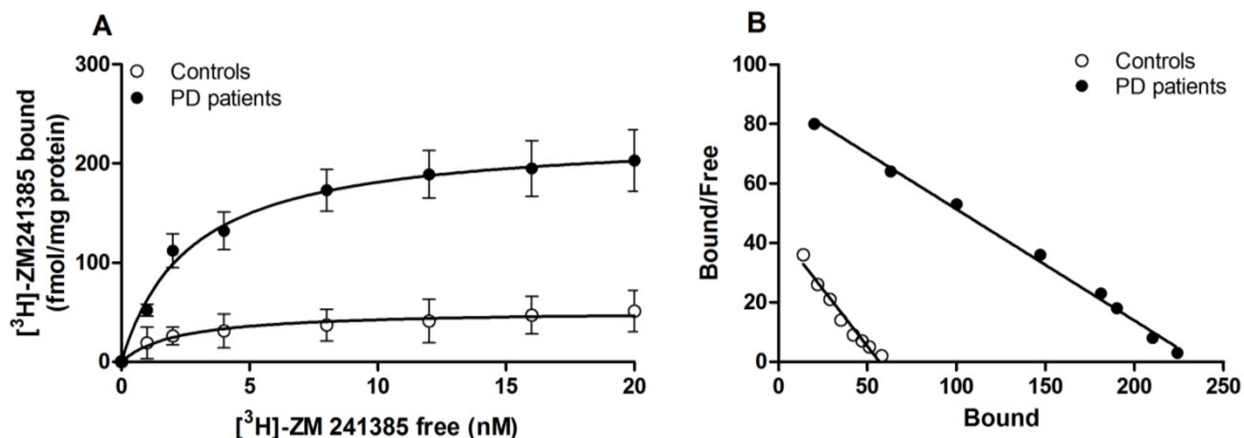
	LYMPHOCYTES			
	Controls		PD Patients	
	K_D (nM)	Bmax (fmol/mg protein)	K_D (nM)	Bmax (fmol/mg protein)
$[^3H]$-DPCPX A_1 AR	1.63±0.11	34±3	1.35±0.06	35±2
	n=10		n=10	
$[^3H]$-ZM 241385 A_{2A} AR	1.32±0.73	59±34	2.81±0.92*	223±93*
	n=89		n=83	
$[^3H]$-MRE2029F20 A_{2B} AR	2.12±0.15	53±4	2.16±0.09	51±3
	n=10		n=10	
$[^3H]$-MRE3008F20 A_3 AR	1.75±0.13	125±8	1.67±0.11	120±5
	n=10		n=10	

Table 2R- Affinity and density of adenosine receptors in lymphocytes of control subjects and PD patients. Data are expressed as means ± SE. T-test, *p < 0.01 vs. control group.

	NEUTROPHILS			
	Controls		PD Patients	
	K_D (nM)	Bmax (fmol/mg protein)	K_D (nM)	Bmax (fmol/mg protein)
[^{3}H]-DPCPX A_1 AR	1.72±0.13	32±3	1.71±0.07	33±2
	n=60		n=10	
[^{3}H]-ZM 241385 A_{2A} AR	1.32±0.08	70±5	1.96±0.09*	160±7*
	n=60		n=56	
[^{3}H]-MRE2029F20 A_{2B} AR	2.41±0.17	78±5	2.51±0.08	82±3
	n=60		n=10	
[^{3}H]-MRE3008F20 A_3 AR	2.32±0.18	436±21	2.57±0.14	424±13
	n=60		n=10	

Table 3R -Affinity and density of adenosine receptors in neutrophils of control subjects and PD patients. Data are expressed as means ± SE. T-test, *p < 0.01 vs. control group.

Lymphocytes



Neutrophils

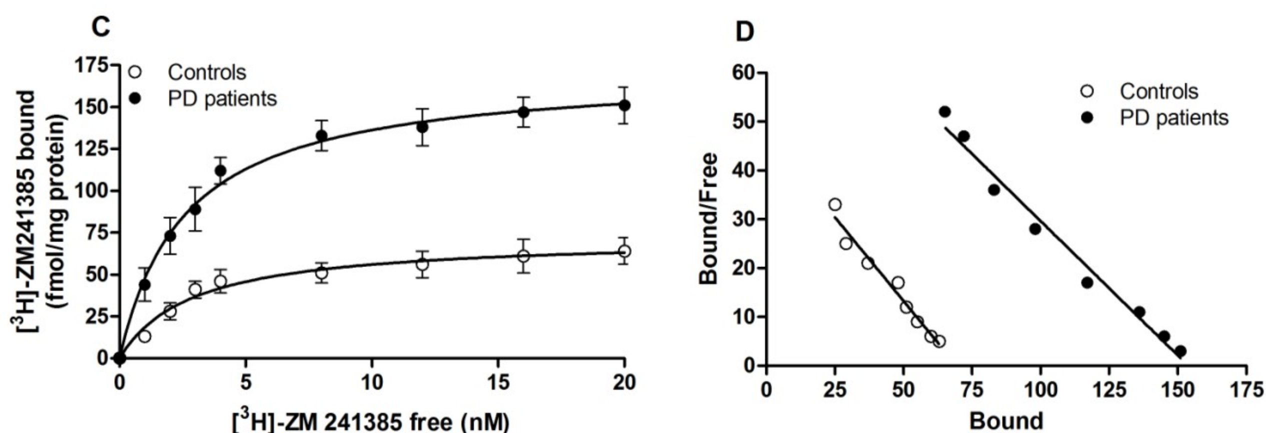


Fig. 4R - Saturation curves an Scatchard plot of [^{3}H]-ZM 241385 binding to A_{2A}ARs on lymphocytes (A, B) and neutrophils (C, D) membranes. Data are expressed as means ± SE. T-test, *p < 0.01 vs. control group.

4. Evaluation of AR mRNA expression and protein level in lymphocytes

AR mRNA in lymphocytes from control subjects and PD patients is reported (Fig. 5R A). The mRNA level of A_{2A}ARs in PD patients was higher when compared with healthy subjects (1.8-fold of increase), while the content of A_1-, A_{2B}-, and A_3AR mRNA in PD (0.8-, 0.9-, and 1.1-fold of increase for A_1, A_{2B}, and A_3ARs, respectively) was not significantly different from healthy subjects, arbitrarily defined as 1. The immunoblot signals of A_{2A}ARs in lymphocytes of controls and PD patients suggests that A_{2A}ARs are differentially expressed in healthy humans and in humans with PD (Fig. 5R B). A_{2A}ARs

proteins are overexpressed in lymphocytes (242%) of PD patients when compared with control subjects.

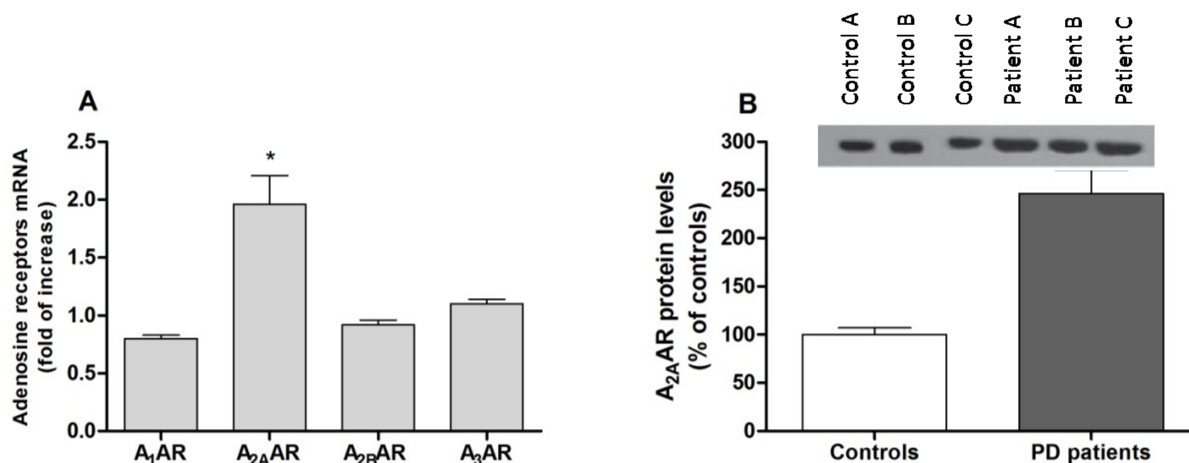


Fig. 5R- A_1-, A_{2A}, A_{2B}-, and A_3R mRNA in PD patients, calculated as ratio of PD patients/healthy subjects (A). Immunoblot and densitometric analysis of A_{2A}ARs in lymphocytes of PD patients and control subjects. Data are expressed as means± SE. T-test, *p <0.01 vs. control group.

5. Effect of adenosine agonists and antagonists on cAMP assays in blood cells

In lymphocytes of PD patients and control subjects, there is no change in basal adenylyl cyclase activity (18±2 and 17±2 pmol/10^6 cells, respectively), in the response of the direct activator forskolin (73±7 and 72±6 pmol/10^6 cells, respectively), and in the presence of the phosphodiesterase inhibitor Ro 201724 (82±8 and 80±7 pmol/10^6 cells, respectively; Fig. 6R). In a cohort of PD patients and control subjects (n=10 for each group), a statistically significant increase of cAMP production by NECA (51±4 and 38±3 pmol/10^6 cells, respectively; $P<0.01$) or CGS 21680 (42±2 and 31±3 pmol/10^6 cells, respectively; $P<0.01$) was found (Fig. 6R). The effect of NECA or CGS 21680 was evaluated in the presence of SCH58261 (1 µM) or caffeine (10 µM), which reduced cAMP levels to control values (in PD patients: 19±2 and 20±2 or 18±2 and 22±2 pmol/10^6 cells, respectively; Fig. 6R). In lymphocytes of PD patients, NECA or CGS 21680 showed EC50 values significantly lower than those in controls, indicating an increased potency of 2.2- to 2.1-fold, respectively (Fig. 7R). Maximal cAMP accumulation is the stimulatory response obtained by maximal concentration of adenosine agonists. In control subjects, NECA (10 µM) and CGS 21680 (10 µM)

mediated an increase of cAMP production reaching values of 100±7 and 112±8 pmol/10^6 cells, respectively.

In PD patients, NECA or CGS 21680 at 5 μM concentrations increased cAMP levels to 98±6 and 105±7 pmol/10^6 cells, respectively.

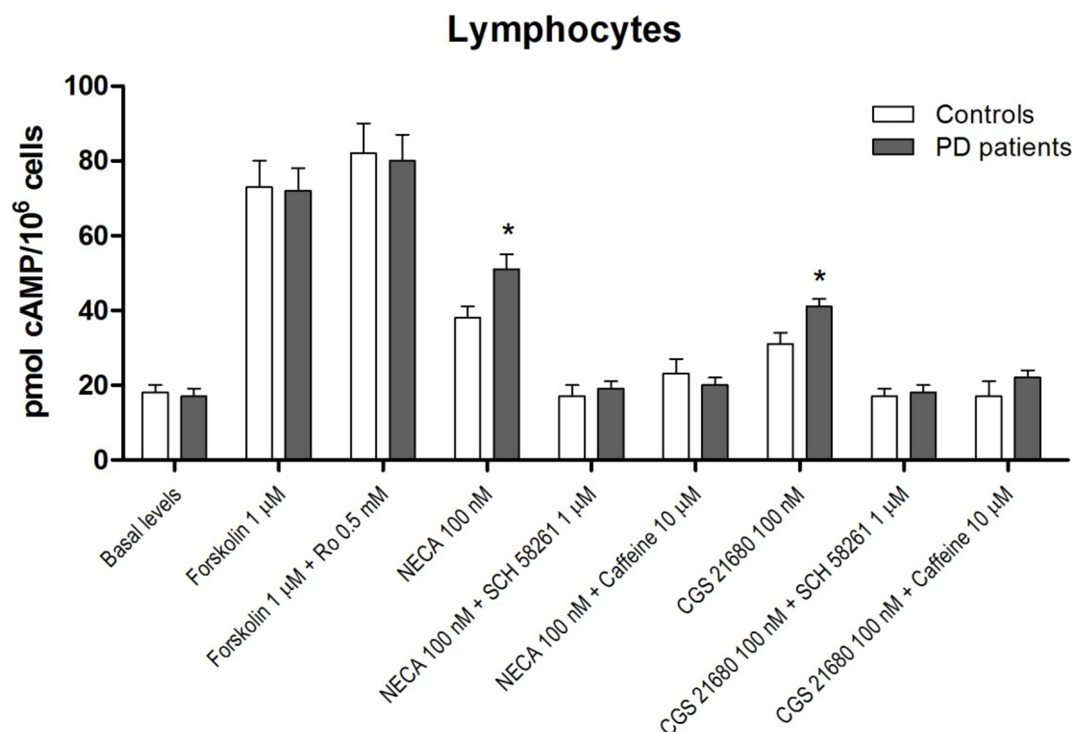


Fig. 6R- Effect of forskolin (1 μM) in the absence and in the presence of Ro 201724 (0.5 mM), NECA (100 nM), and CGS 21680 (100 nM) in the absence and in the presence of SCH 58261 (1 μM) and caffeine (10 μM) on the stimulation of cAMP levels in lymphocytes obtained from controls (n=10) and PD patients (n=10). Data are expressed as means ± SE. 1-way ANOVA with Dunnett's *post hoc* test, *$p < 0.01$ vs. basal levels.

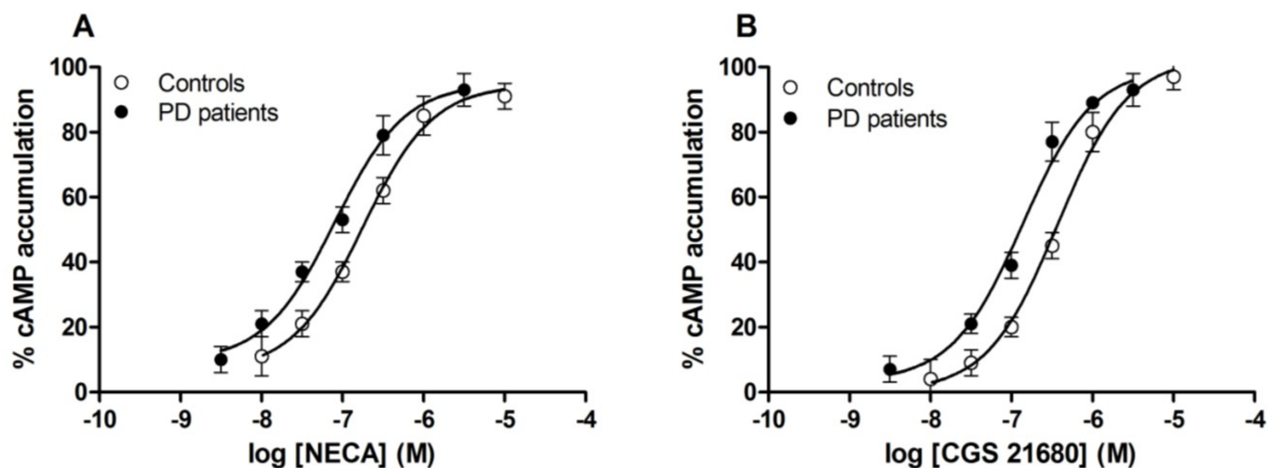


Fig. 7R- NECA (A) and CGS 21680 (B) concentration response curves on cAMP accumulation in lymphocytes obtained from controls (n=10) and PD patients (n=10). Data are expressed as means ± SE.

6. TNF-α concentration in plasma from PD patients

Basal levels of TNF-α were measured in plasma from control subjects (31±3 pg/ml) and PD patients (92±8 pg/ml). A marked increase of TNF-α concentration was observed in plasma PD patients when compared with healthy volunteers (Fig. 7R).

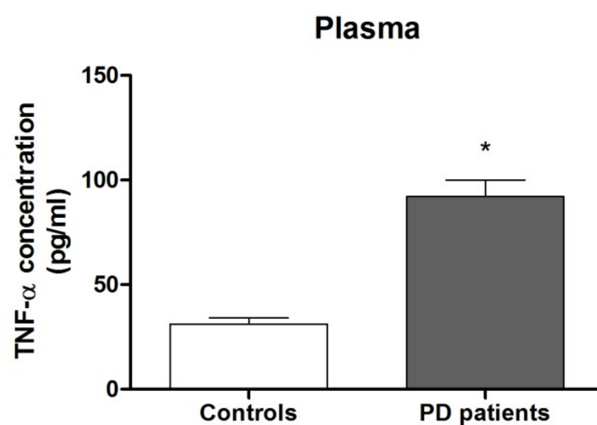


Fig. 7R- TNF-α levels in plasma of the examined subjects (n=6 for each group). Data are expressed as means ± SE. T-test, *$p < 0.01$ vs. control group.

7. Adenosine concentration in human plasma from PD patients

Preliminary experiments were conducted to calibrate the potentiostat and the associated sensors. A buffer containing various concentrations of adenosine was used to evaluate the change in current from the baseline and to obtain a calibration curve with a high correlation coefficient (r=0.99).

Figure 8R shows the concentration of adenosine and adenosine metabolites represented by inosine and hypoxanthine in control subjects and PD patients. Adenosine metabolite concentrations in the micromolar range were significantly higher in PD patients (5.1±0.4 μM) than in controls (2.0±0.2 μM).

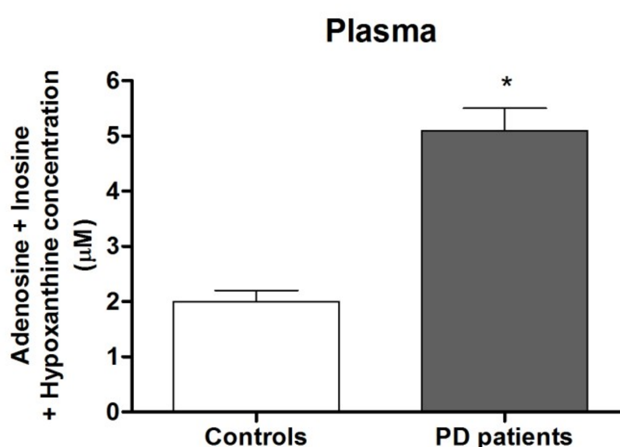


Fig. 8R- Adenosine, inosine, and hypoxanthine concentrations in plasma of the examined subjects (n= 6 for each group). Data are expressed as means ± SE. T-test, *p < 0.01 vs. control group.

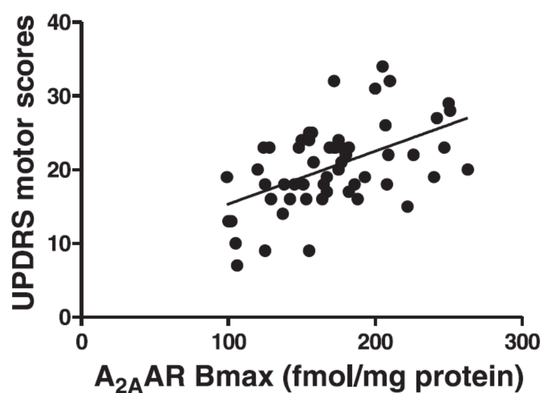
8. Correlation between A_{2A}AR binding parameters or functional data and clinical characteristics

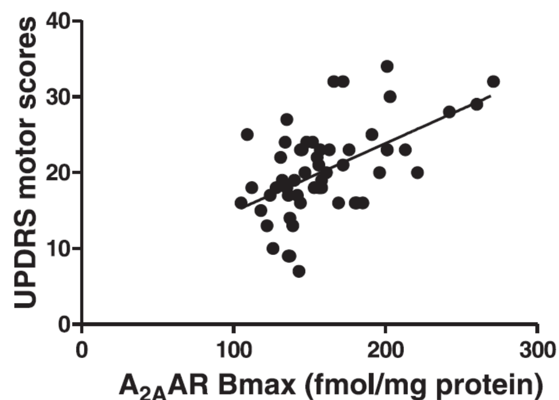
In our statistical analysis no differences in A_{2A}AR parameters across different age, age at onset or disease duration was found. Correlation analyses show that Bmax and K_D of the A_{2A}AR correlate with severity of PD symptoms. A direct correlation was found between UPDRS motor score and A_{2A}AR binding parameters (Bmax and K_D) and an inverse correlation with functional data (EC_{50}) in lymphocytes and neurophils of PD patients (table 4R; fig. 9R). A very high correlation between UPDRS (total score and part I, II, III, and IV score) and A_{2A}AR density was detected. The strongest correlation

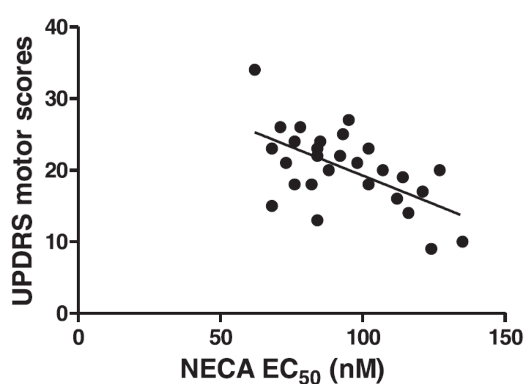
was found with UPDRS III: the correlation coefficient with Bmax reaches a value of $r=0.515$ ($p<0.0001$) and of $r=0.58$ ($p<0.0001$) with K_D. Although $A_{2A}AR$ density and affinity don't appeare to increase with increasing Hoehn and Yahr stage, ($p=0.14$ and $p=0.09$ respectively). The correlation between UPDRS part IV score and $A_{2A}AR$ binding parameters was stronger when considering only items 32-39 that assessed dyskinesias and motor fluctuations. MMSE that concern the global cognitive status show a negative correlation with Bmax values ($r=-0.25$) and the affinity of the $A_{2A}AR$ decreased with decreasing MMSE score ($r=-0.42$). Any significant correlation was found with GDS and FAB score (table 5R). Variable that were found to be significantly related to Bmax and K_D values were submitted in multiple regression analyses confirming the relationships showed by the previous analysis (Table 6R). In PD patients with dyskinesias the $A_{2A}AR$ density was significantly higher ($p<0.005$) and the affinity lower ($p<0.0001$) than in patients without these complications (Bmax=265±102 and 187±70 fmol/mg protein respectively). Patients with motor fluctuations have $A_{2A}AR$ density significantly higher and a lower affinity.

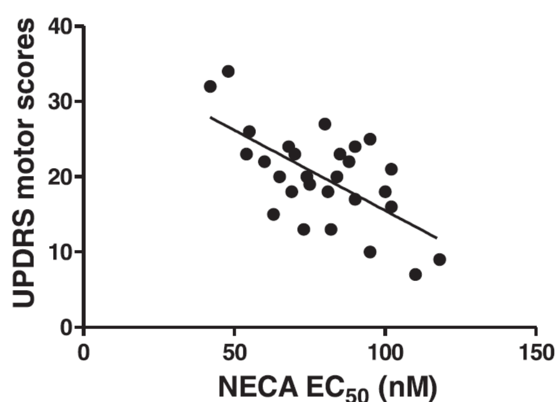
The multivariate logistic regression analysis shows that patients above the upper quartile had an increased odds to suffer from dyskinesias as compared to patients in the lowest quartile while patients with Bmax above the median had a 3.7 times higher odds to suffer from dyskinesias. Similar results were found when analyzing the relationship between Bmax and motor fluctuations.

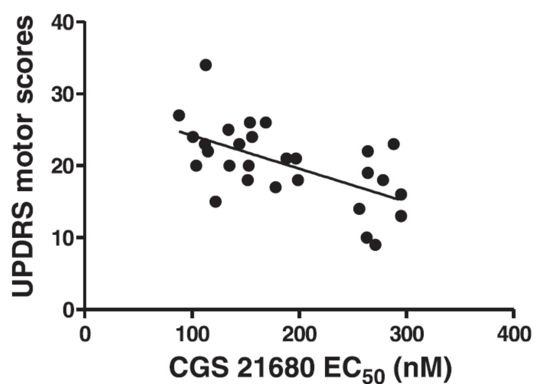
LYMPHOCYTES | **NEUTROPHILS**











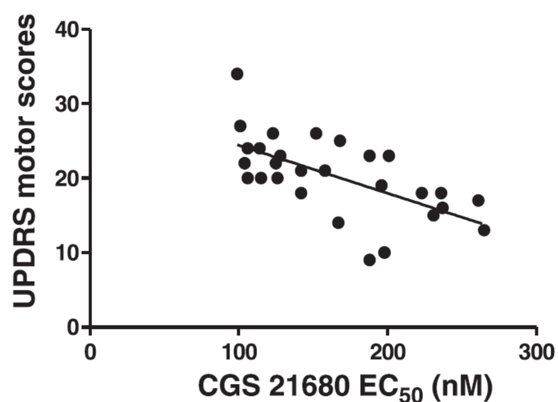


Fig. 9R - Linear regression analysis between UPDRS motor score and maximum number of A_{2A}ARs (Bmax) in lymphocytes and neutrophils. Similar correlation was also evaluated between UPDRS motor score and A_{2A}AR agonists such as NECA and CGS 21680 potency (EC_{50}) in lymphocytes and neutrophils.

A_{2A}AR parameters		UPDRS motor score Linear regression		UPDRS motor score Spearman coefficient	
		r	*P*-value	ρ	*P*-value
LYMPHOCYTES	**Bmax (fmol/mg protein)**	0.5162	<0.0001	0.4725	0.0002
	EC_{50} (NECA, nM)	0.5919	0.0009	-0.4916	0.0079
	EC_{50} (CGS 21680, nM)	0.5940	0.0009	-0.5584	0.0020
NEUTROPHILS	**Bmax (fmol/mg protein)**	0.5429	<0.0001	0.4837	0.0002
	EC_{50} (NECA, nM)	0.6407	0.0002	-0.5049	0.0061
	EC_{50} (CGS 21680, nM)	0.6265	0.0004	-0.6673	0.0001

Table 4R- Correlation between UPDRS motor score and A_{2A}ARs density (Bmax) or functional (EC_{50}) parameters in lymphocytes and neutrophils.

Demographic and PD Characteristics	Bmax r	K_D r
Age	0.185	0.096
Age at onset	0.16	0.033
Duration since onset	0.08	0.123
UPDRS I	**0.42 (P<0.0001)**	**0.31 (P<0.005)**
UPDRS II	**0.48 (P<0.0001)**	**0.397 (p<0.0001)**
UPDRS III	**0.515 (P<0.0001)**	**0.58 (P<0.0001)**
UPDRS IV	**0.326 (P>0.005)**	**0.35 (P<0.001)**
UPDRS IV*	**0.49 (P<0.0001)**	**0.48 (P<0.001)**
UPDRS Total	**0.53 (p<0.0001)**	**0.55 (P<0.0001)**
MMSE	**-0.25 (P<0.005)**	**-0.42 (P<0.0001)**
FAB	-0.212	-0.17
GDS	0.18	0.143
LEDD	0.25	-0.01

Table 5R- Correlation analysis between clinical variables and A_{2A}AR affinity (K_D) or density (Bmax). R, Pearson correlation coefficient; UPDRS IV*, item 32-39.

Clinical variable	Bmax, fmol/mg protein Beta (P value)	Kd, nM Beta (P value)
UPDRS I	0.39 (<0.0001)	0.31 (<0.001)
UPDRS II	0.44 (<0.0001)	0.35 (<0.005)
UPDRS III	0.46 (<0.0001)	0.47 (<0.0001)
UPDRS IV	0.30 (<0.0001)	0.23 (<0.05)
UPDRS IV (items 32-39)	0.38 (<0.0001)	0.29 (<0.005)
UPDRS total	0.49 (<0.0001)	0.46 (<0.0001)
MMSE	-0.3 (<0.01)	-0.35 (<0.005)

Table 6R - Regression coefficients (multiple regression analysis) between $A_{2A}AR$ affinity (K_D, nM) and density (Bmax, fmol/mg protein) and clinical variable.

	Dyskinesias OR (95% CI)*	Motor fluctuations OR (95% CI)*
Bmax upper vs lowest quartile	16.9 (4-71.3)	6.4 (1.9-21.5)
Bmax above the median vs lowest quartile	3.7 (1.4-9.8)	3.2 (1.1-8.8)
Kd upper vs lowest quartile	3.86 (1.2-12.5)	1.95 (0.9-5.37)
Kd above the median vs lowest quartile	2.4 (1.04-5.88)	1 (0.39-2.6)

Table 7R - Multivariate logistic regression analyses of binding parameters and dyskinesia/motor fluctuations. *OR adjusted for age, disease duration and LEDD.

9. Adenosine and dopamine binding experiments in PC12 cells

The presence of A_1-, A_{2A}-, A_{2B}-, and A_3ARs and D_2DRs in untreated or NGF-treated PC12 cells is shown (Fig. 10R). NGF treatment did not modify the affinity and/or density of ARs or DRs. The effect of TNF-α in A_{2A}AR saturation binding experiments revealed a significant increase of A_{2A}AR density of 2-fold in untreated or NGF-treated PC12 cells (Bmax=7.8±0.7 fmol/10^6 cells and 15.8±1.6 fmol/10^6 cells respectively before and after TNF-α treatment; Fig. 11R). This proinflammatory cytokine did not affect the affinity and density of D_2DRs (Fig. 11R). In untreated or NGF-treated PC12 cells, CGS 21680 (1 μM) mediated a statistically significant decrease of A_{2A}AR and D_2DR density. Interestingly, the presence of SCH 58261 (1 μM) mediated an up-regulation of A_{2A}ARs and D_2DRs of 1.8- to 1.9-fold and 1.5- to 1.6-fold in comparison with controls, respectively (Fig. 12R).

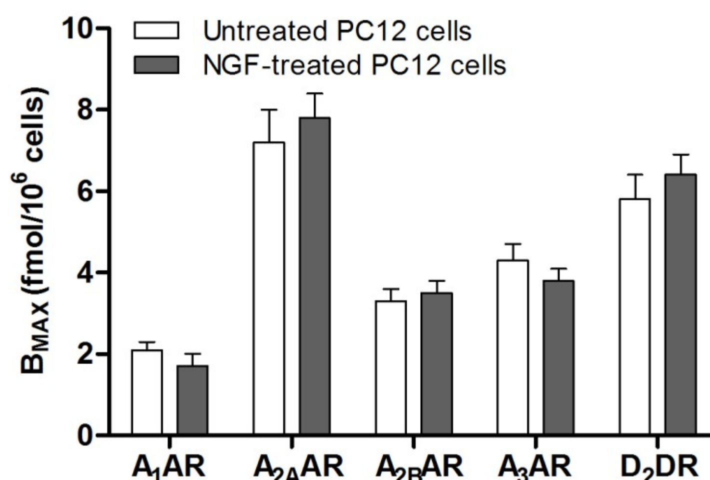


Fig. 10R- A_1-, A_{2A}-, A_{2B}-, and A_3AR density (Bmax, fmol/10^6 cells) in untreated or NGF-treated PC12 cells. Data are expressed as means±SE.

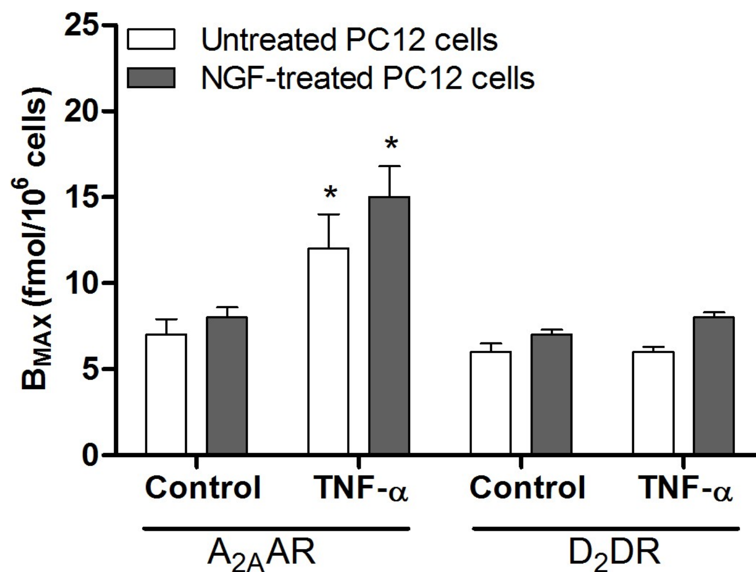


Fig. 11R- Effect of TNF-α (100 ng/ml) on $A_{2A}AR$ density in untreated or NGF-treated PC12 cells. Data are expressed as means ± SE. T-test, *p <0.01 vs. control group.

	Untreated PC12 cells		**NGF-treated PC12 cells**	
	[^{3}H]-ZM241385 $A_{2A}AR$	[^{3}H]-Spiperone D_2DR	[^{3}H]-ZM241385 $A_{2A}AR$	[^{3}H]-Spiperone D_2DR
Control	K_D=1.85±0.19 nM Bmax=7.2±0.8 fmol/10^6 cells	K_D=0.72±0.08 nM Bmax=6.0±0.6 fmol/10^6 cells	K_D=1.84±0.21 nM Bmax=7.8±0.7 fmol/10^6 cells	K_D=0.76±0.06 nM Bmax=6.5±0.5 fmol/10^6 cells
TNF-α treatment 100 ng/ml	K_D=1.95±0.16 nM Bmax=14.6±1.3* fmol/10^6 cells	K_D=0.72±0.06 nM Bmax=6.2±0.6 fmol/10^6 cells	K_D=1.91±0.18 nM Bmax=15.8±1.6* fmol/10^6 cells	K_D=0.68±0.07 nM Bmax=6.7±0.6 fmol/10^6 cells
CGS21680 1µM	K_D=1.88±0.17 nM Bmax=4.1±0.4* fmol/10^6 cells	K_D=0.71±0.08 nM Bmax=3.7±0.4* fmol/10^6 cells	K_D=1.93±0.19 nM Bmax=4.4±0.4* fmol/10^6 cells	K_D=0.74±0.07 nM Bmax=3.9±0.3* fmol/10^6 cells
SCH58263 1µM	K_D=1.87±0.18 nM Bmax=12.8±1.1* fmol/10^6 cells	K_D=0.76±0.08 nM Bmax=9.2±0.6 fmol/10^6 cells	K_D=1.85±0.17 nM Bmax=14.9±1.7* fmol/10^6 cells	K_D=0.73±0.06 nM Bmax=10.6±0.8* fmol/10^6 cells

Table 8R- Effect of TNF-α, CGS21680 and SCH58261 in PC12 cells on affinity and density of $A_{2A}ARs$ and D_2DRs. Data are expressed as mean ± SE. T-test, *p <0.01 vs. control group.

10. cAMP assays in PC12 cells

In untreated or NGF-treated PC12 cells, forskolin increased basal cAMP levels of 3.1- and 3.0-fold, respectively. The modulation of cAMP production by the adenosine agonists NECA and CGS 21680 was investigated in the absence and in the presence of TNF-α (Fig. 12R). NECA and CGS 21680 increased the basal adenylyl cyclase activity by 2.1- and 1.9-fold, respectively. The presence of TNF-α mediated a significant increase of NECA- and CGS 21680-stimulated cAMP production at the 100 nM concentration in untreated or NGF-treated PC12 cells (Fig. 12R). In PC12 cells, NECA and CGS 21680 showed a high potency in cAMP production, with an EC_{50} of 115 ± 12 and 159 ±14 nM, respectively. The pretreatment of TNF-α caused an increase of NECA and CGS 21680 potency (EC_{50} 63±6 and 78±8 nM, respectively; $p<0.01$ *vs.* control condition) that correlated closely with the increase in A_{2A}AR density. Analogous results were also obtained in NGF-treated PC12 cells (Fig. 13R). The selective A_{2A}AR antagonist SCH 58261 (1 µM) completely abrogated the stimulatory effect of adenosine agonists. Similarly, caffeine (10 µM) reduced NECA or CGS 21680-induced cAMP stimulation in NGF treated PC12 cells before and after TNF-α treatment (Fig. 12R). In NGF-treated PC12 cells, NECA (10 µM) and CGS 21680 (10 µM) mediated an increase of cAMP production reaching values of 98± 6 and 95± 5 pmol/10^6 cells, respectively. After treatment with TNF-α, NECA or CGS 21680 at 5 µM increased cAMP production to 106 ± 7 and 100 ± 6 pmol/10^6 cells, respectively (Fig. 13R A, B).

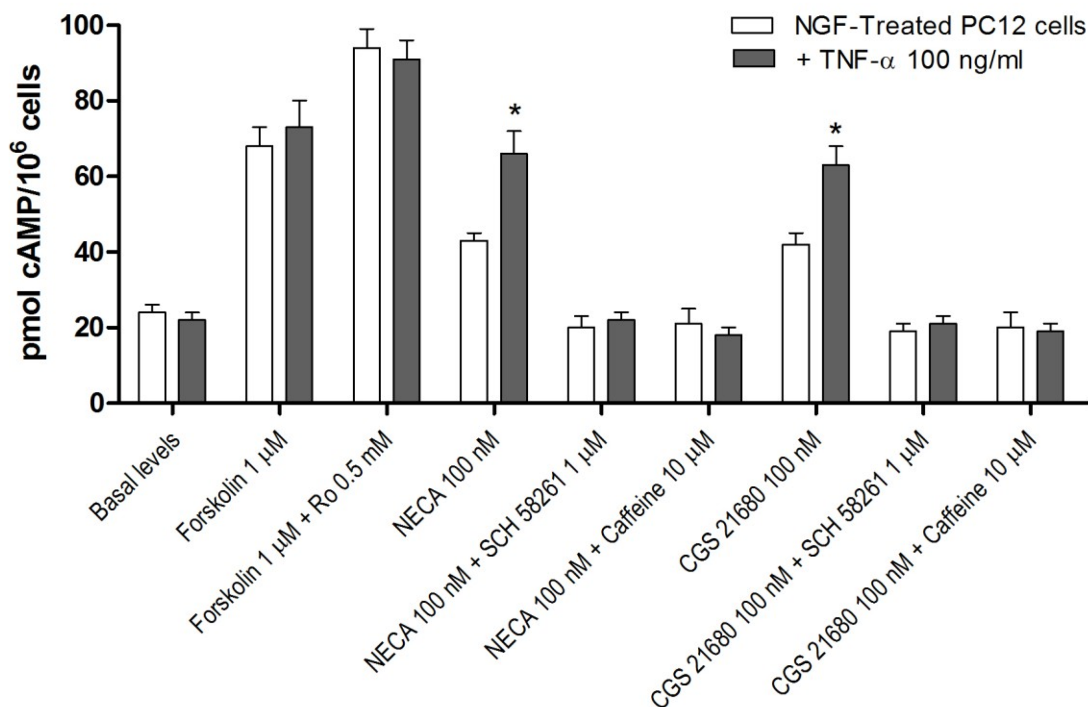


Fig. 12R- Effect of forskolin (1 µM) in the absence and in the presence of Ro 201724 (0.5 mM), NECA (100 nM), and CGS 21680 (100 nM) in the absence and in the presence of SCH 58261 (1 µM) and caffeine (10 µM) on the stimulation of cAMP levels in NGF-treated PC12 cells before and after TNF-α treatment. Data are expressed as means ± SE. 1-way ANOVA with Dunnett's *post hoc* test, *p < 0.01 vs. basal levels.

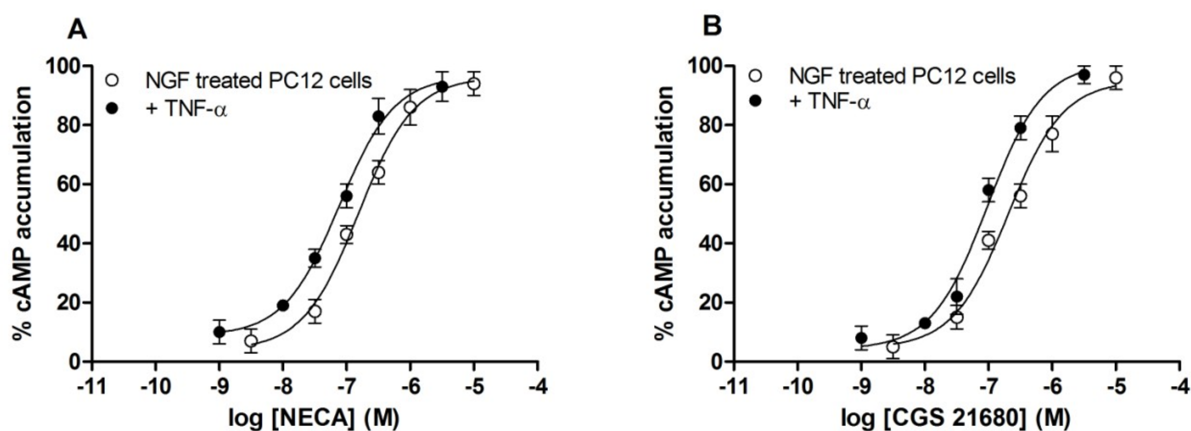


Fig.13R- NECA (A) and CGS 21680 (B) concentration response curves on cAMP accumulation in NGF treated PC12 cells before and after TNF-α treatment. Data are expressed as means ± SE.

Effect of A_{2A} agonist/antagonist on dopamine uptake in PC12 cells

Dopamine uptake in PC12 cells was investigated in the absence and in the presence of NECA (1 μM) and CGS21680 (1 μM). A_{2A}AR agonists caused a statistically significant increase of dopamine uptake of 1.6- to 1.7-fold in comparison with untreated PC12 cells. Similarly, in NGF-treated PC12 cells, NECA and CGS 21680 increased dopamine uptake by 62 and 70%, respectively. A selective A_{2A}AR antagonist (SCH 58261, 10 μM) blocked the increased dopamine uptake induced by the adenosine agonists. Similarly, caffeine (10 μM) abrogated NECA and CGS 21680 stimulatory effects (Fig. 14R A, B).

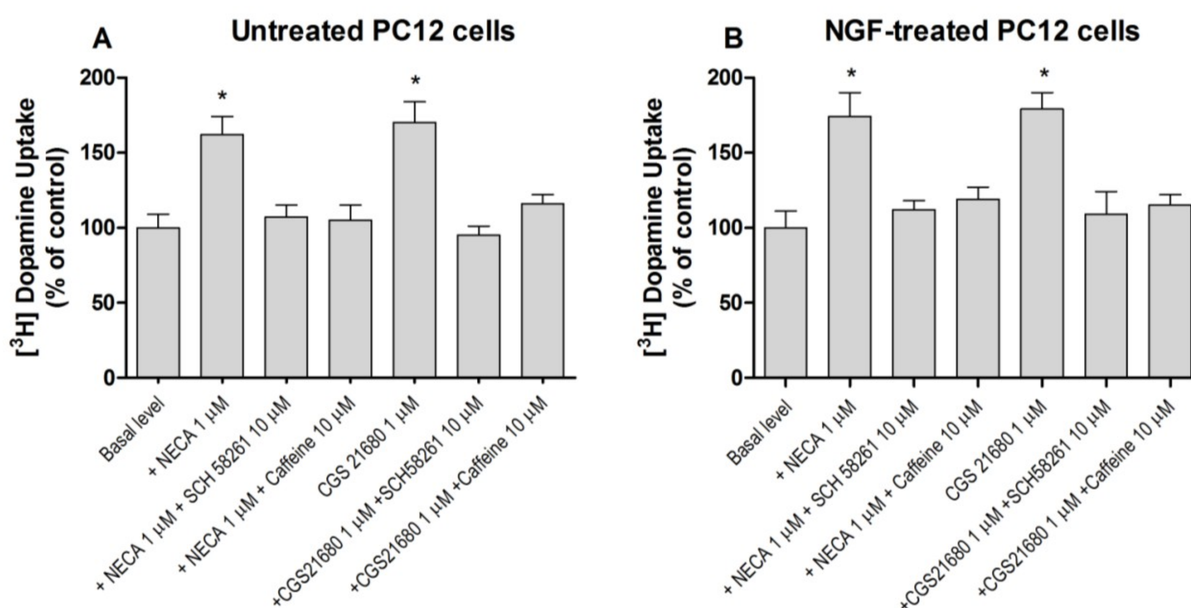


Fig. 14R - Dopamine uptake in the absence and in the presence of typical adenosine agonists NECA (1 μM) and CGS 21680 (1 μM) or of adenosine antagonists SCH 58261 (10 μM) and caffeine (10 μM) in untreated (A) or NGF-treated (B) PC12 cells. Data are expressed as means ± SE. 1-way ANOVA with Dunnett's *post hoc* test, *p < 0.01 vs. basal levels.

CHAPTER V

DISCUSSION AND CONCLUSIONS

DISCUSSION AND CONCLUSIONS

Chronic administration of L-DOPA, the gold standard treatment for PD, leads to abnormal motor responses known as dyskinesias involving involuntary choreic or dystonic movements in 50% of patients in 5 years after the initiation of treatment [163]. Fifty years after its introduction, L-DOPA remains the most effective form of oral symptomatic treatment for motor symptoms. There are three form of dyskinesias that commonly occur with L-DOPA use: peak dose dyskinesia, diphasic dyskinesia at the end of L-DOPA efficacy and off-period dystonia when a patient receives subtherapeutic levels of L-DOPA [164]. Actually researchers are focused on the study of A_{2A}AR like an alternative therapeutic target to use it alone or in combination to L-DOPA with the purpose to avoid side effects. These researchers are supported by an important evidence of A_{2A}AR involvement in PD due to an antagonistic effects on D_2DRs [165].

From our study emerges an increase of A_{2A}ARs on putamen membranes associated to an increase of mRNA levels and proteins in respect to the putamen of control subjects. However there is any difference in D_2DRs expression in PD patients putamen compare to controls. This data confirm the key role of A_{2A}ARs in neurological diseases and are conform with previous studies demonstrating the selective distribution of A_{2A}AR mRNA in the caudate-putamen and the increase of A_{2A}AR transcript in PD patients. However literature data are in contrast with our binding results because did are not found any differences in [^{3}H]-CGS21680 binding site to A_{2A}AR in the striatum and pallidum of PD patients when compared with controls. A possible explanation of these differences could be due to the characteristics of radioligand used (agonist versus antagonist). In CHO cells stably transfected with the human A_{2A}AR, the Bmax value, calculated using the antagonist [^{3}H] CGS21680, was twice as high as that found when was used the agonist [^{3}H] SCH58261, because the agonist radioligand only binds to receptors present in the high-affinity state [166]. With this premise we sustain that agonist radioligand label only the active adenosine binding site hampering the identification of all A_{2A}ARs. As a consequence, it has been suggested that in human tissues adenosine antagonists are better for characterizing A_{2A}AR of both active and inactive status. Our experimental data reveal that the increased A_{2A}ARs density is associated with and enhanced stimulation of cAMP production suggesting an increase of the active form of the receptor. Moreover the evaluation of adenosine concentration and its metabolites in human plasma shows that adenosine levels are increase in PD patients. There are many reports in literature showing that

the physiological effects of adenosine, which include regulation of cytokines production, are mediated by binding to cell surface ARs regulating the anti-inflammatory response.

In previous studies elevated levels of proinflammatory cytokines such as TNF-α in the striatum of PD brains have been found. Enhanced expression of TNF-α has been shown in the cerebrospinal fluid as well as in the basal ganglia of PD patients. The presence of elevated levels of cytokines seems to amplify and sustain the neuroinflammation as well as further consequent immune responses leading to irreversible destruction of dopaminergic neurons.

How expected TNF-α levels in plasma of PD patients that we analyzed are much more elevated than in healthy controls suggesting an inflammatory status in PD.

TNF-α is able to increase $A_{2A}AR$ mRNA and proteins in different A549 lung epithelial cell line [167], peripheral blood mononuclear cells [168] and polymorphonuclear neutrophils [169]. Then we suppose that high levels of TNF-α are able to drive the overexpression of $A_{2A}AR$ in brain of PD patients.

To investigate the role of TNF-α in $A_{2A}AR$ expression we use pheochromocytoma cells (PC12), a widely useful cell line for the study of neurobiological mechanisms. No difference were found for ARs and D_2DR in untreated or NGF-treated PC12 cells, suggesting that the treatment for the cell differentiation did not modify the receptor binding and functional parameters. TNF-α in PC12 cells determined an increase of $A_{2A}ARs$ but not D_2DRs, suggesting that high levels of this cytokine could be one of the causes of $A_{2A}AR$ upregulation in PD. In addition TNF-α mediates an increase in $A_{2A}AR$ functionality, as demonstrated by cAMP production: intensified antagonistic interaction with dopaminergic signaling could be explain by the opposite effect exerted on second messenger production.

The direct involvement of $A_{2A}AR$ in dopamine uptake was investigated. CGS21680 and NECA, selective and unselective $A_{2A}AR$ agonists, increased dopamine uptake, reducing the dopamine concentration active in the receptor sites. A good agreement was found between cAMP accumulation and dopamine uptake, suggesting that the effect of adenosine agonists could be mediated by a molecular pathway involving cAMP. Inhibition of dopamine uptake in PD could provide symptoms improvement, an effect that could be mediated by $A_{2A}AR$ antagonists.

We focused our research also in the identification of a possible new biomarker for PD diagnosis and disease progression. In our study we found a strong correlation between $A_{2A}AR$ alteration, in lymphocytes and neutrophils, and PD severity as indexed by UPDRS motor score: PD patients having an higher UPDRS motor score also exhibit an increase in $A_{2A}AR$

density. This suggest that the disease severity is closely associated with the increase of $A_{2A}AR$. Our data revealed also an high inverse correlation between cognitive performance as approximated by the MMSE and $A_{2A}AR$ density and affinity.

Interestingly we found a significantly different $A_{2A}AR$ profile in PD patients with and without levodopa-induced motor complications independent on age, disease duration and medication.

Studies on animal models of dyskinesia show that $A_{2A}AR$ plays a critical role in the development and persistence of complications induced by repeated levodopa treatments. From experiments in 6-OHDA lesioned mice, WT and $A_{2A}AR$-KO, treated with a low dose of L-DOPA, emerges that $A_{2A}AR$ is required for the development and persistence of L-DOPA-induced behavioral sensitization [163]. Moreover the increase of $A_{2A}AR$ mRNA (+60%) was found in dyskinetic patients compared with non-dyskinetic PD patients in the lateral putamen [153].

These results demonstrate that $A_{2A}AR$ antagonist could be an useful target for PD therapy by inhibiting dopamine uptake representing a novel pharmacological strategy based on the A_{2A}/D_2 interaction.

In our opinion a sustained exposition to an inflammatory stimuli and a consequent massive release of TNF-α in the brain could induce an hyper-expression of $A_{2A}ARs$ by activation of the nuclear factor NF-kB, mediating the well known effect on D_2DRs also through an antagonism on the second messenger production and cAMP production. This effect and the increase of dopamine up-take after $A_{2A}AR$ stimulation (with a consequent reduction of dopamine to receptorial sites), could be the mechanism mediating the motor impairment in PD patients.

According to National Institutes of Health a biomarker is a “characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. A good molecular biomarker for PD could help to identify at-risk individuals, to detect the disease at early stages, improving diagnostic accuracy and prognosis [170]. In our study $A_{2A}AR$ alterations in peripheral blood cells demonstrated several characteristics that make them attractive as a possible biomarker of PD. They can be evaluated through a non-invasive blood examination, easily acceptable even for repeated tests. Moreover the relationship between $A_{2A}AR$ density and affinity seems to be independent from demographic characteristics, disease duration and antiparkinsonian drugs.

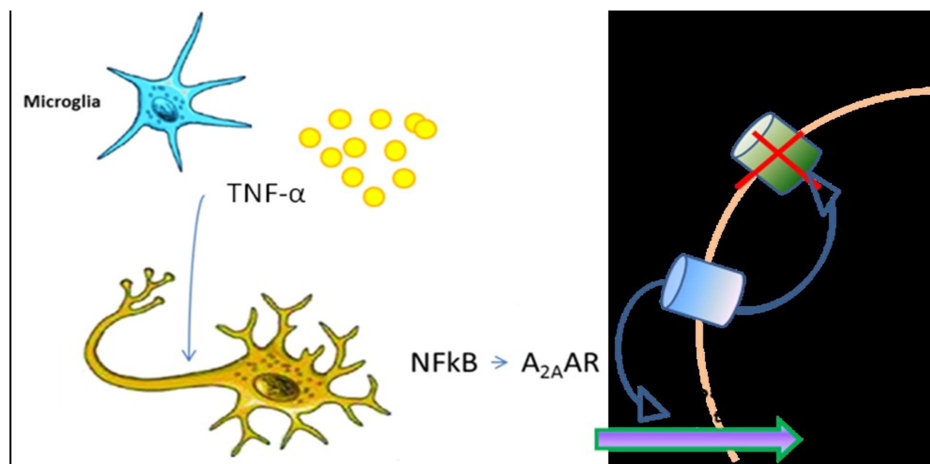
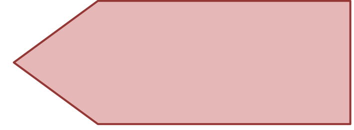


Fig. 1D – Possible A_{2A}AR molecular mechanism inducing motor impairment.



SUPPLEMENTS

UPDRS

Sezione I
ATTI VITA' PSICHICA, COMPORTAMENTO E TONO DELL'UMORE

Alterazione delle facoltà intellettuali
0. Nessuna.
1. Lieve dimenticanza consistente in ricordo parziale di
accadimenti, senza altre difficoltà.
2. Modesta perdita di memoria, con disorientamento e moderata difficoltà a trattare problemi complessi. Lieve ma moderato peggioramento delle funzioni domestiche con necessità di suggerimento occasionale.
3. Perdita di memoria severa con disorientamento nel tempo e spesso nello spazio. Peggioramento severo a trattare problemi.
4. Perdita di memoria severa con orientamento conservato solo alla persona. Incapacità ad emettere giudizi o a risolvere problemi. Richiede molto aiuto nella cura della persona. Non può essere lasciato da solo.

Alterazioni del pensiero
0. Nessuna.
1. Immaginazione fervida.
2. Allucinazioni "benigne" con interiorizzazione.
3. Allucinazioni o delusioni da occasionali a frequenti; senza interiorizzazione, possibile interferenza col quotidiano.
4. Allucinazioni permanenti, delusione o psicosi floride. Non in grado di badare a sé stessi.

Depressione
0. Assente.
1. Periodi di tristezza odi senso di colpa maggiori del normale, non sostenuto mai per giorni o settimane.
2. Depressione sostenuta (i settimana o più).
3. Depressione sostenuta con sintomi vegetativi (insonnia, anoressia, calo ponderale, caduta dell'interesse).
4. Depressione sostenuta con sintomi vegetativi e pensieri o tentativi suicidi.

Motivazione/iniziativa
0. Normale.
1. Meno affermativa del normale.
2. Perdita dell'iniziativa o disinteresse in attività elettive (non routinarie).
3. Perdita dell'iniziativa o disinteresse nel quotidiano (routine).
4. Isolamento, completa perdita della motivazione.

Sezione II
ATTIVITÀ DI VITA QUOTIDIANA (DETERMINARE PER FASI ON/OFF)

Linguaggio
0. Normale.
1. Lievemente interessato; nessuna difficoltà ad essere compreso.
2. Moderatamente interessato; qualche volta richiesta la
ripetizione delle affermazioni.
3. Severamente interessato; frequentemente richiesta la
ripetizione delle affermazioni.
4. Incomprensibile per la maggior parte del tempo.

Salivazione
0. Normale.
1. Lieve ma definito eccesso di saliva nella bocca; può presentare sbavamento notturno.
2. Saliva moderatamente in eccesso; può presentare sbavamento minimo.
3. Marcato eccesso di saliva con qualche sbavamento.
4. Marcato sbavamento, richiede costante ripulimento.

Deglutizione
0. Normale.
1. Soffocamento raro.
2. Soffocamento occasionale.
3. Richiede cibi delicati.
4. Richiede alimentazione attraverso sondino naso gastrico o gastrostomia.

Scrittura
0. Normale.
1. Leggermente lenta o piccola.
2. Moderatamente lenta o piccola; tutte le parole sono leggibili.
3. Gravemente colpita; non tutte le parole sono leggibili.
4. La maggior parte delle parole non sono leggibili.

Taglio dei cibi e manualità degli utensili
0. Normale.
1. Piuttosto lento e goffo, ma non necessita di aiuto.
2. Può tagliare la maggior parte dei cibi, anche se goffamente e lentamente; necessita di qualche aiuto.
3. I cibi devono essere tagliati da qualcuno. ma può ancora alimentarsi lentamente.
4. Necessità di essere alimentati.

Il vestirsi
0. Normale.
1. Piuttosto lento, ma non necessita di aiuto.
2. Assistenza occasionale nell'abbottonamcnto e nell'infilare le braccia nelle maniche.
3. Richiesto aiuto considerevole, ma può compiere qualche movimento da solo
4.incapacità

Igiene personale
0. Normale.
1. Piuttosto lenta, ma non necessita di aiuto.
2. Necessita di aiuto per la doccia o il bagno; molto lenta
l'igiene personale.
3. Assistenza richiesta per le operazioni di lavaggio: usare lo spazzolino per i denti, asciugatura dei capelli, andare in bagno
4. Cateteri di Foley o altri supporti meccanici.

Girarsi nel letto o aggiustarsi le coperte
0. Normale.
1. Piuttosto lento e goffo, ma non necessita di aiuto.
2. Può girarsi da solo o aggiustarsi le coperte, ma con grande difficoltà.
3. Può tentare, ma non girarsi o aggiustarsi le coperte da solo.
4. Incapacità.

Caduta (non collegata a freezing)
0. Nessuna.
1. Cadute rare.
2. Cadute occasionali, meno di una al giorno.
3. Cadute, in media una al giorno.
4. Cadute, molte più di una al giorno.

Freezing durante la marcia
0. Nessuna.
1. Freezing raro durante la marcia; può avere avvio esitante.
2. Freezing occasionale durante la marcia.
3. Freezing frequente; occasionalmente caduta per freezing.
4. Cadute frequenti per freezing.

Marcia
0. Normale.
1. Leggera difficoltà; può non oscillare le braccia o può trascinare le gambe.
2. Difficoltà moderata, ma richiede poca o nessuna assistenza.
3. Grave disturbo del moto, richiede assistenza.
4. Non può completamente camminare, anche con assistenza.

Tremore
0. Assente
1. Fine ed infrequentemente presente.
2. Moderato; fastidioso per il paziente.
3. Severo; interferisce con molte attività.
4. Marcato; interferisce con la maggior parte delle attività.

Disturbi sensoriali collegati ai parkinsonismi
0. Nessuno.
1. Occasionalmente presenta intorpidimetnto, formicolio
o lieve dolore.
2. Frequentemente presenta intorpidimento, formicolio o
dolore; non angoscioso.
3. Frequente sensazione dolorosa.
4. Dolore straziante.

Sezione III
ESAME DELLA MOTRICITÀ
Linguaggio
0. Normale.
1. Lieve perdit;~ dell'espressione, della dizione e/o del
volume.
2. Monotono. disarticolato ma comprensibile; moderatamente peggiorato.
3. Marcato peggioramento, difficoltà a comprendere.
4. Incomprensibile

Espressione del volto
0. Normale.
1. lpomimia minimale, potrebbe essere normale amimia.
2. Lieve ma definita diminuzione dell'espressione facciale.
3. Moderata ipomimia; labbra dischiuse per buona parte del tempo.
4. Volto immobile a maschera, con severa o completa perdita dell'espressività del volto; labbra dischiuse poco o molto.

Tremore a riposo
0. Assente.
1. Fine ed infrequentemente presente.
2. Leggero nell'ampiezza e persistente. Oppure moderato nell'ampiezza ma solo intermittentemente presente.
3. Moderato nell'ampiezza e presente per la maggior parte del tempo.
4. Marcato nell'ampiezza e presente per la maggior parte del tempo.

Attività o tremore posturale delle mani
0. Assente.
1. Fine; presente con l'attività.
2. Moderato nell'ampiezza, presente con l'attività.

3. Marcato, ma l'intero range dei movimenti si compi con facilità.
4. Severo, il range dei movimenti si compie con difficoltà.

Rigidità (valutata sui movimenti passivi delle articolazioni maggiori a paziente rilassato in posizione seduta. Ignorare i movimenti a scatti)
0. Assente.
1. Leggera o identificabile solo quando attivata allo specchio o da altri movimenti.
2. Da lieve a moderata.
3. Marcata, ma range completo di motricità compiuto facilmente.
4. Severa, range di motricità compiuto con difficoltà.

Picchiettamento delle dita (il paziente picchietta il pollice contro il dito indice in rapida successione con la massima ampiezza possibile, separatamente per ciascuna mano)
0. Normale
1. Leggermente rallentato e/o ridotta l'ampiezza.
2. Moderatamente indebolito. Determinato e rapidamente
faticoso. Può presentare arresto occasionale del movimento.
3. Severamente indebolito. Frequente esitazione nell'iniziare il movimento oppure arresto del movimento in corso.
4. Può a malapena eseguire il compito.

Movimento delle mani (il paziente apre *e* chiude le mani in rapida successione con la massima ampiezza possibile,separatamente per ciascuna mano)
0. Normale.
1. Leggermente rallentato e/o ridotta l'ampiezza.
2. Moderatamente indebolito. Determinato e rapidamente faticoso. Può presentare arresto occasionale del movimento.
3. Severamente indebolito. Frequente esitazione nell'iniziare il movimento oppure arresto del movimento in corso.
4. Può a malapena eseguire il compito.

**Rapidi movimenti alternati delle mani (movimenti di pronazione-supinazione delle mani, verticalmente o orizzontalmente, con un'ampiezza tanto larga quanto possibile, con
entrambe le mani simultaneamente)**
0. Nornali.
1. Leggermente rallentati e/o ridotta l'ampiezza.
2. Moderatamente indeboliti. Determinato e rapidamente faticoso. Può presentare arresto occasionale del movimento.
3. Severamente indeboliti. Frequente esitazione nell'iniziare il movimento oppure arresto del movimento in corso.
4. Può a malapena eseguire il compito.

Agilità delle gambe (il paziente batte il tallone sul pavimento in rapida successione, alzando l'intera gamba. L'ampiezza dovrebbe essere di circa 8 cm)
0. Normale.
1. Leggermente rallentata e/o ridotta l'ampiezza.
2. Moderatamente indebolita. Determinata e rapidamente faticosa. Può presentare arresto occasionale del movimento.
3. Severamente indebolita. Frequente esitazione nell'iniziare il movimento oppure arresto del movimento in corso.
4. Può a malapena eseguire il compito.

Alzarsi dalla sedia (il paziente tenta di alzarsi da una sedia a schienale dritto di legno o di metallo, con le braccia incrociate sul torace)
0. Normale.
1. Lento o può necessitare di più di un tentativo.
2. Si dà la spinta mediante i braccioli della sedia
3. Tende a ricadere indietro e può dover tentare più di una volta, ma può alzarsi senza aiuto.
4. Incapace di alzarsi senza aiuto.

Postura
0. Normale eretta.
1. Non completamente eretta, postura leggermente chinata; potrebbe essere normale per le persone anziane.
2. Postura moderatamente chinata, francamente anormale; può essere moderatamente inclinata su di un lato.
3. Postura severamente chinata con cifosi; può essere moderatamente inclinata su di un lato.
4. Marcata flessione con estrema anormalità della postura.

Andatura
0. Normale.
1. Cammina lentamente, può trascinarsi a piccoli passi, ma senza festinazione o spinta.
2. Cammina coh difficoltà, ma richiede poca o nessuna assistenza; pùò presentare qualche festinazione, passi piccoli o spinta.
3. Severo disturbo dell'andatura; richiede assistenza.
4. Non può camminare affatto, eventualmente con assistenza.

Stabilità posturale (risponde al rapido spostamento indietro prodotto dalla trazione sulle spalle a paziente eretto con gli occhi aperti ed i piedi lievemente distanziati)
0. Normale.
1. Retropulsione, ma compensa senza aiuto.
2. Assenza di risposta posturale; potrebbe cadere se non sorretto dall'esaminatore.
3. Molto instabile, tende a perdere l'equilibrio spontaneamente.
4. Incapace di rimanere in piedi senza assistenza.

Bradicinesia ed ipocinesia corporea (combinante lentezza, esitazione, diminuita oscillazione delle braccia, ampiezza ridotta e povertà dei movimenti in generale)
0. Nessuna.
1. Lentezza minimale, conferendo movimento ad un carattere voluto, potrebbe essere normale per alcune persone. Possibilmente ridotta ampiezza.
2. Lieve grado di lentezza e povertà di movimenti che è francamente anormale. Alternativamente, una certa riduzione d'ampiezza.
3. Moderata lentezza, povertà o limitata ampiezza del movimento.
4. Marcata lentezza, povertà o limitata ampiezza del movimento.

Sezione IV
COMPLICANZE DELLA TERAPIA
(NELLA SETTIMANA TRASCORSA)

A. DISCINESIE

Durata: in che percentuale si presentano le discinesie durante lo stato di veglia? (informazioni anamnestiche)
0. Nessuna.
1. 1—25% della giornata.
2. 26—50% della giornata.
3. 51—75% della giornata.
4. 76-100% della giornata.

Invalidità: quanto invalidanti sono le discinesie? (informazioni anamnestiche; possono essere modificate attraverso l'accertamento d'ufficio)
0. Non invalidanti.
1. Lievemente invalidanti.
2. Moderatamente invalidanti.
3. Severamente invalidanti.
4. Completamente invalidanti.

Discinesie dolorose: quanto dolorose sono le discinesie?
0. Assenza di discinesie dolorose.
1. Lievi.
2. Moderate
3. Severe.
4. Marcate.

Presenza di distonia mattutina (notizie anamnestiche)
0. No
1. Si

B OSCILLAZIONE CLINICA

Ci sono dei periodi prevedibili di "off" come a determinare il tempo successivo ad una dose di farmaco?
0. No.
1. SÌ.

Ci sono dei periodi imprevedibili di "off" come a determinare il tempo successivo ad una dose di farmaco?
0. No.
1. Sì.

I periodi di "off" insorgono rapidamente? (per esempio in pochi secondi)
0. No.
1. Sì.

In quale percentuale il paziente presenta uno stato di "off"nello stato di veglia?
0. Nessuna.
1. 1-25% della giornata.
2. 26-50% della giornata.
3. 51-75% della giornata.
4. 76-100% della giornata.

C ALTRE COMPLICANZE

Il paziente presenta anoressia, nausea o vomito?
0. No
1. sì.

Il paziente presenta qualche disturbo del sonno? (per esempio, insonnia o ipersonnia)
0. No.
1. Sì.

Il paziente presenta ortostatismo sintomatico?
0. No.
1. Sì.

Allegato 2 HOEHN AND YAHR SCALE

STADIO 0
Nessun segno di malattia;

STADIO 1
Coinvolgimento unilaterale;

STADIO 1,5
Coinvolgimento unilaterale assiale (rigidità del collo);

STADIO 2
Coinvolgimento bilaterale senza alterazioni dell'equilibrio;

STADIO 2,5
Coinvolgimento bilaterale con instabilità posturale ma capacità di riprendere l'equilibrio quando spinto;

STADIO 3
Coinvolgimento bilaterale da lieve a moderato, instabilità posturale con incapacità di riprendere l'equilibrio se spinto;

STADIO 4
Disabilità grave, il paziente può ancora camminare o stare in piedi da solo ma è gravemente disabile;

STADIO 5
Il paziente è allettato o in sedie a rotelle se non aiutato.

Allegato 3 MMSE

1) ORIENTAMENTO TEMPORO-SPAZIALE	Il paziente sa riferire:	
	ANNO	☐
	STAGIONE	☐
	MESE	☐
	GIORNO DEL MESE	☐
MAX PUNTI 5	GIORNO DELLA SETTIMANA	☐
	Il paziente sa riferire:	
	STATO	☐
	REGIONE	☐
	CITTÀ	☐
	LUOGO	☐
MAX PUNTI 5	PIANO IN CUI SI TROVA	☐

2) MEMORIA DI REGISTRAZIONE	Il medico pronuncia ad alta voce (una sola volta) il nome di 3 oggetti (CASA, GATTO, PANE) al ritmo di uno al secondo.	
	Il paziente deve ripeterli una prima volta.	☐
	Fa ripetere nuovamente al paziente la sequenza delle 3 parole fino a quando non le abbia imparate.	☐
MAX. PUNTI 3		

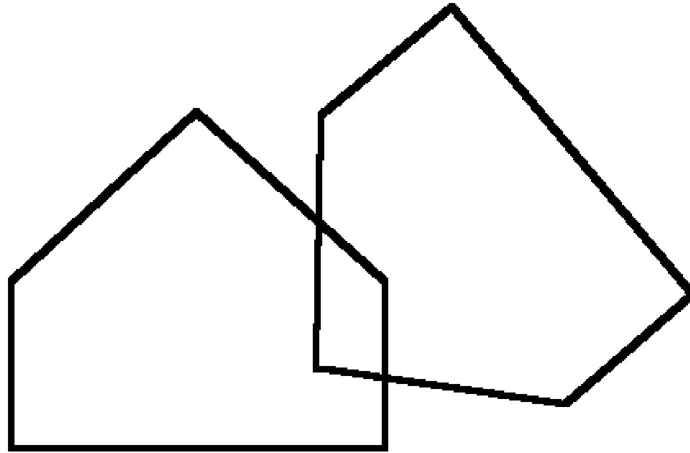
3) ATTENZIONE CALCOLO	Sottrazione seriale di 7 da 100 (93-86-79-72-65)	
	Fermarsi dopo le prime cinque risposte.	☐
MAX. PUNTI 5	Se il paziente avesse difficoltà di calcolo, far scandire lettera per lettera la parola "MONDO" all'indietro (ODNOM).	☐

4) MEMORIA DI RIEVOCAZIONE	Richiamare i 3 termini precedentemente imparati.	☐
MAX.PUNTI 3		

5) LINGUAGGIO	Il paziente deve riconoscere un oggetto.	
	"Come si chiama questa?" (indicando una MATITA)	☐
	"Come si chiama questo?"(indicando un OROLOGIO)	☐
	Ripeta "SOPRA LA PANCA LA CAPRA CAMPA" 1 P.TO	☐
	Esecuzione di un COMPITO su comando:	
	a) Con la mano destra prenda questo foglio 1 P.TO	☐
	b) lo pieghi a metà 1 P.TO	☐
	c) lo appoggi sulle sue ginocchia 1 P.TO	☐
	Presentare al paziente un foglio con la seguente scritta "CHIUDA GLI OCCHI". Il paziente deve obbedire all'ordine	☐
MAX PUNTI 8	Fare scrivere al paziente una FRASE formata almeno da soggetto e verbo 1 P.TO	☐

6) PRASSI VISUOCOSTRUTTIVA

Far copiare al paziente il DISEGNO



MAX 1 PUNTO

Allegato 4 FAB

1. **Somiglianza** (concettualizzazione)
- *In che cosa sono simili:*
-*una banana e un'arancia*
(In caso di fallimento totale: "non sono simili" o di fallimento parziale: "entrambe hanno la buccia", aiutare il paziente : *" la banana e l'arancia sono entrambe …."*; ma assegnare 0 a questa risposta; non aiutare i paziente per i due successivi item)
- *un tavolo e una sedia?*
- *Un tulipano, una rosa e una margherita?"*
- punteggio: solo le risposte categoriali (frutta, mobili, fiori) sono considerate corrette.
3 risposte corrette 3
2 risposte corrette 2
1 risposta corretta 1
nessuna risposta corretta 0

2. **Fluenza fonemica** (flessibilità mentale)
- *"Dica il maggior numero possibile di parole che cominciano con la lettera "S", qualsiasi parola eccetto cognomi o nomi propri".*
Se il paziente non dà nessuna risposta nei primi 5 secondi, dire: *"Per esempio, serpente".* Se il paziente si ferma per oltre 10 secondi, stimolarlo dicendo : *"Qualsiasi parola che cominci con la lettera "S" .* La prova dura 60 secondi.
- Punteggio: ripetizioni o variazioni (scarpa, scarpone), cognomi o nomi propri non sono contate come risposte corrette.
Più di 9 parole 3
Da 6 a 9 parole 2
Da 3 a 5 parole 1
Meno di 3 parole 0

3. **Serie Motorie** (programmazione)
- *"Guardi con attenzione quello che faccio".*
L'esaminatore seduto di fronte al paziente effettua tre volte, da solo, con la mano sinistra la serie di Luria "pugno-taglio-piatto".
- *"Ora faccia lo stesso, con la mano destra prima con me poi da solo".*
L'esaminatore effettua tre volte la stessa serie con il paziente, poi gli dice : *"continui da solo"*
- Punteggio:
- il paziente effettua da solo, correttamente, 6 serie consecutive 3
- il paziente effettua da solo, correttamente, almeno 3 serie consecutive 2
- il paziente sbaglia da solo, ma effettua correttamente almeno 3 serie 1 consecutive con l'esaminatore
- il paziente non riesce ad effettuare 3 serie consecutive neppure 0 con l'esaminatore.

4. **Istruzioni contrastanti** (sensibilità all'interferenza)
- *"Batta due volte quando io batto una volta".*
Per essere sicuri che il paziente abbia capito le istruzioni, si effettua una serie di tre prove : 1-1-1.
- *"Batta una volta quando io batto due volte".*Per essere sicuri che il paziente abbia capito le istruzioni, si effettua una serie di tre prove :2-2-2.
L'esaminatore effettua la serie seguente : 1-1-2-1-2-2-2-1-1-2.

- punteggio:
- nessun errore 3
- 1 o 2 errori 2
- più di 2 errori 1
- il paziente batte come l'esaminatore per almeno 0
4 prove consecutive

5. **Go – No - Go** (controllo inibitorio)
- *"Batta una volta quando io batto una volta".* Per essere sicuri che il paziente abbia capito le istruzioni, si effettua una serie di tre prove : 1-1-1.
- *"Non batta quando io batto due volte".* Per essere sicuri che il paziente abbia capito le istruzioni, si effettua una serie di tre prove : 2-2-2-2.
L'esaminatore effettua la serie seguente : 1-1-2-1-2-2-2-1-1-2.
- punteggio:
- nessun errore 3
- 1 o 2 errori 2
- più di 2 errori 1
- il paziente batte come l'esaminatore per almeno 0
4 prove consecutive

6. **Comportamento di prensione** (autonomia ambientale)

- L'esaminatore è seduto di fronte al paziente. Mettere le mani del paziente con le palme in alto, appoggiate sulle ginocchia. Senza dire nulla e senza guardare il paziente, l'esaminaotre porte le sue mani vicino a quelle del paziente e ne tocca le palme, contemporaneamente da ambo i lati, osservando se il paziente spontanemaente le afferra. Se il paziente le afferra, l'esaminatore prova di nuovo dopo avergli detto: *"Non prenda le mie mani"*
- punteggio :
- il paziente non afferra le mani dell'esaminatore 3
- il paziente e vita o chiede cosa deve fare 2
- il paziente afferra le mani senza esitazione 1
- il paziente afferra le mani dell'esaminatore 0
anche dopo che gli ha chiesto di non farlo

TOTALE …../18

Geriatric Depression Scale

	SI NO
1 Siete sostanzialmente soddisfatti della vostra vita?	0 1
2 Avete abbandonato molte delle vostre attività e interessi?	1 0
3 La vita vi sembra vuota?	1 0
4 Siete spesso annoiati?	1 0
5 Avete speranza nel futuro?	0 1
6 Siete infastiditi da pensieri che non riuscite a toglervi dalla testa?	1 0
7 Siete di buon umore per la maggior parte del tempo?	0 1
8 Avete paura che qualcosa di brutto stia per accadervi?	1 0
9 Vi sentite felice per la maggior parte del tempo?	0 1
10 Vi sentite spesso senza speranza?	1 0
11 Vi sentite spesso irrequieto e agitato?	1 0
12 Preferite rimanere in casa piuttosto che uscire e fare cose nuove?	1 0
13 Il futuro vi preoccupa?	1 0
14 Vi sembra di avere dei problemi di memoria più degli altri?	1 0
15 Vivere, per voi, è meraviglioso?	0 1
16 Vi sentite spesso giù di corda e malinconico?	1 0
17 Vi sentite inutile nella vostra situazione?	1 0
18 Vi tormentate molto pensando al passato?	1 0
19 Trovate la vita molto interessante?	0 1
20 È difficile per voi iniziare delle nuove cose?	1 0
21 Vi sentite carichi di energie?	0 1
22 Vi sembra che la vostra situazione sia senza speranza?	1 0
23 Pensate che la maggior parte delle persone stia meglio di voi?	1 0
24 Siete spesso agitati per cose poco importanti?	1 0
25 Avete frequentemente voglia di piangere?	1 0
26 Avete difficoltà a concentrarvi?	1 0
27 È un piacere per voi alzarvi al mattino?	0 1
28 Preferite evitare di incontrare gente?	1 0
29 Vi riesce facile prendere delle decisioni?	0 1
30 Vi sembra che la vostra mente sia lucida come prima?	1 0

QUESTIONARIO SULLA QUALITÀ DELLA VITA DEI MALATI DI PARKINSON

COGNOME E NOME ________________________________ ANNI ______

DATA DI NASCITA ________

A causa del morbo di parkinson, quanto spesso si è trovato/a nelle seguenti situazioni, nel mese scorso?
La preghiamo di segnare una sola casella per ogni domanda.

1. **Ha avuto difficoltà nello svolgere attività di tempo libero come avrebbe voluto?**
   - ☐ MAI
   - ☐ OCCASIONALMENTE
   - ☐ QUALCHE VOLTA
   - ☐ SPESSO
   - ☐ SEMPRE O NON CI RIESCE AFFATTO

2. **Ha avuto difficoltà nello svolgere attività domestiche, p.e. bricolage, faccende domestiche, cucinare?**
   - ☐ MAI
   - ☐ OCCASIONALMENTE
   - ☐ QUALCHE VOLTA
   - ☐ SPESSO
   - ☐ SEMPRE O NON CI RIESCE AFFATTO

3. **Ha avuto difficoltà nel portare le borse della spesa?**
   - ☐ MAI
   - ☐ OCCASIONALMENTE
   - ☐ QUALCHE VOLTA
   - ☐ SPESSO
   - ☐ SEMPRE O NON CI RIESCE AFFATTO

4. **Ha avuto problemi nel camminare per circa un chilometro?**
   - ☐ MAI
   - ☐ OCCASIONALMENTE
   - ☐ QUALCHE VOLTA
   - ☐ SPESSO
   - ☐ SEMPRE O NON CI RIESCE AFFATTO

5. **Ha avuto problemi nel camminare per circa 100 metri?**
   - ☐ MAI
   - ☐ OCCASIONALMENTE
   - ☐ QUALCHE VOLTA
   - ☐ SPESSO
   - ☐ SEMPRE O NON CI RIESCE AFFATTO

6. **Ha avuto problemi nel muoversi per casa senza difficoltà come avrebbe voluto?**
   - ☐ MAI
   - ☐ OCCASIONALMENTE

- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

7. **Ha avuto difficoltà nel muoversi in luoghi pubblici?**
   - ☐ MAI
   - ☐ OCCASIONALMENTE
   - ☐ QUALCHE VOLTA
   - ☐ SPESSO
   - ☐ SEMPRE O NON CI RIESCE AFFATTO

8. **Ha avuto bisogno dell'aiuto di qualcuno per uscire di casa?**
   - ☐ MAI
   - ☐ OCCASIONALMENTE
   - ☐ QUALCHE VOLTA
   - ☐ SPESSO
   - ☐ SEMPRE O NON CI RIESCE AFFATTO

9. **Ha avuto paura o si è preoccupato/a di poter cadere quando si è trovato/a in pubblico?**
   - ☐ MAI
   - ☐ OCCASIONALMENTE
   - ☐ QUALCHE VOLTA
   - ☐ SPESSO
   - ☐SEMPRE O NON CI RIESCE AFFATTO

10. **È stato/a costretto/a a restare in casa più di quanto avesse desiderato?**
    - ☐ MAI
    - ☐ OCCASIONALMENTE
    - ☐ QUALCHE VOLTA
    - ☐ SPESSO
    - ☐ SEMPRE O NON CI RIESCE AFFATTO

11. **Ha avuto difficoltà nel lavarsi?**
    - ☐ MAI
    - ☐ OCCASIONALMENTE
    - ☐ QUALCHE VOLTA
    - ☐ SPESSO
    - ☐ SEMPRE O NON CI RIESCE AFFATTO

12. **Ha avuto difficoltà nel vestirsi?**
    - ☐ MAI
    - ☐ OCCASIONALMENTE
    - ☐ QUALCHE VOLTA
    - ☐ SPESSO
    - ☐ SEMPRE O NON CI RIESCE AFFATTO

13. **Ha avuto problemi nell'abbottonare i vestiti o allacciarsi le scarpe?**
    - ☐ MAI
    - ☐ OCCASIONALMENTE
    - ☐ QUALCHE VOLTA
    - ☐ SPESSO
    - ☐ SEMPRE O NON CI RIESCE AFFATTO

14. **Ha avuto problemi nello scrivere in modo chiaro?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

15. **Ha avuto difficoltà nel tagliare il cibo?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

16. **Ha avuto difficoltà nel tenere in mano il bicchiere senza versarne il contenuto?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

17. **Si è sentito/a depresso/a?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

18. **Si è sentito/a isolato/a e abbandonato/a?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

19. **Ha avuto voglia di piangere o è stato/a sul punto di farlo?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

20. **Si è sentito/a pieno/a di rabbia o risentimento?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

21. **Si è sentito/a ansioso/a?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA

- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

22. **Si è sentito/a preoccupato/a per il suo futuro?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

23. **Ha voluto cercare di nascondere agli altri la propria malattia?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

24. **Ha cercato di evitare occasioni che le richiedevano di mangiare o bere in pubblico?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

25. **Ha provato imbarazzo in pubblico a causa del morbo di Parkinson?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

26. **Si è sentito/a preoccupato/a a causa della reazione degli altri nei suoi confronti?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

27. **Ha avuto problemi nelle relazioni personali con le persone che le sono vicine (amici intimi, parenti stretti)?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

28. **Le è mancato il sostegno da parte del coniuge o compagno/a?**
Se non ha coniuge o campagno/a la preghiamo di fare un segno in questa casella
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

29. **Le è mancato il sostegno da parte di familiari o amici intimi?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

30. **Si è addormentato/a improvvisamente durante il giorno?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

31. **Ha avuto problemi di concentrazione, p.es. leggendo o guardando la TV?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

32. **Ha avuto l'impressione di avere problemi di memoria?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

33. **Ha fatto sogni angosciosi o ha avuto allucinazioni?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

34. **Ha avuto difficoltà nel parlare ed esprimersi?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

35. **Si è sentito/a incapace di comunicare correttamente con gli altri?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

36. **Si è sentito/a ignorato/a dagli altri?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA

- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

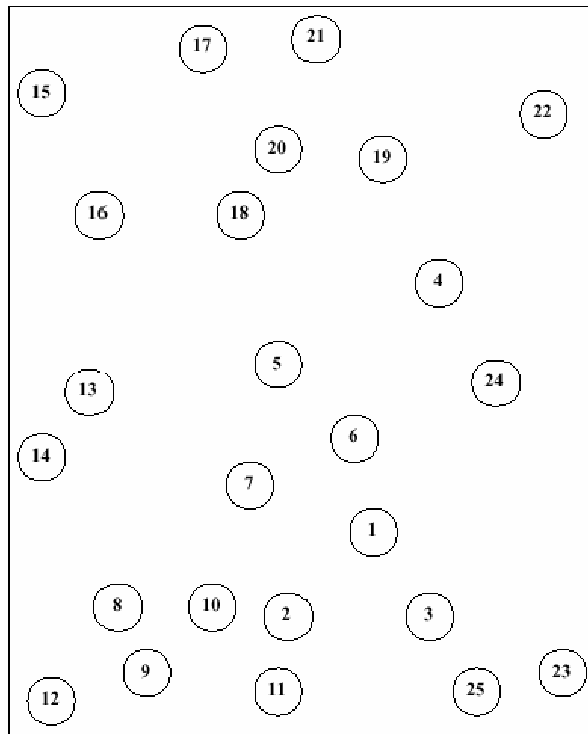
37. **Ha avuto crampi o spasmi muscolari dolorosi?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

38. **Ha avuto dolori alle articolazioni o ad altre parti del corpo?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

39. **Ha provato una spiacevole sensazione di caldo o freddo?**
- ☐ MAI
- ☐ OCCASIONALMENTE
- ☐ QUALCHE VOLTA
- ☐ SPESSO
- ☐ SEMPRE O NON CI RIESCE AFFATTO

Allegato 7 TMT-A

Trail Making Test Part A



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Publications:

1. *Effect of pulsed electromagnetic field exposure on adenosine receptors in rat brain*. Varani K, Vincenzi F, Targa M, **Corciulo C**, Fini M, Setti S, Cadossi R, Borea PA. Bioelectromagnetics, 2011, 33: 279-287

2. *The anti-tumor effect of A3 adenosine receptors is potentiated by pulsed electromagnetic fields in cultured neural cancer cells.* Vincenzi F, Targa M, **Corciulo C**, Gessi S, Merighi S, Setti S, Cadossi R, Borea PA, Varani K. PLoS One., 2012, 7(6):e39317.

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Conference presentations:

- *Effect of pulsed electromagnetic field exposure on adensoine receptors in human fibroblast-like synoviocytes.* Varani K, Vincenzi F, **Corciulo C**, Masieri F, Ongaro A, De Mattei M, Massari L, Setti S, Borea PA, Cadossi R. 10th International Conference of the European Bioelectromagnetics, Roma, 21st–24th February 2011.

- *The anti-tumor effect of A_3 adenosine receptors is potentiated by pulsed electromagnetic fields in cultured neural cancer cells*. **Corciulo C**, Vincenzi F, Targa M, Gessi S, Merighi S, Setti S, Cadossi R, Borea PA, Varani K. 16° Seminario SIF , Rimini 17th -19th September 2012.

- *The stimulation of A_3 adenosine receptors reduces bone-residing breast cancer in a rat preclinical model*. Targa M, Vincenzi F, **Corciulo C**, Paradiso B, Parrilli A, Fini M, Lanza G, Borea PA, Varani K. 16° Seminario SIF , Rimini 17th -19th September 2012.

- *Adenosine A_{2A} receptor as a potential new therapeutic target for the prevention/treatment of osteoarthritis.* **Corciulo C**, Mediero A, Wilder T, Cronstein BN. ACR/ARHP-Annual Meeting, San Diego 25th-30th October 2013.

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