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ALTERED EXPRESSION AND FUNCTIONALITY OF

A$_{2A}$ ADENOSINE RECEPTORS IN HUNTINGTON'S

DISEASE AND OTHER POLYGLUTAMINE DISORDERS

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Contents

GENERAL INTRODUCTION

ADENOSINE

Adenosine is a nucleoside composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) via a β-N9-glycosidic bond (Figure 1).
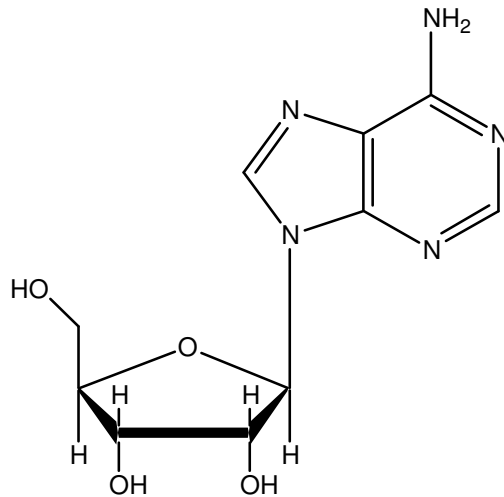
Figure 1 – Chemical structure of Adenosine

Adenosine is an endogenous nucleoside-signalling molecule, which, by acting on specific membrane receptors produces a number of physiological and pathophysiological effects in both the central nervous system and peripheral organs. Under normal conditions, adenosine is continuously formed intracellularly as well as extracellularly. The intracellular production is mediated either by an intracellular 5'-nucleotidase, which dephosphorylates or by hydrolysis of S-adenosyl-homocysteine (Fredhoml et al., 2001). Adenosine generated intracellularly is transported into the extracellular space mainly via specific bi-directional transporters through facilitated diffusion that efficiently evens out the intra- and extracellular levels of adenosine. The dephosphorylation of extracellular AMP to adenosine, mediated by ecto-5'-nucleotidase, is the last step in the enzymatic chain that catalyzes the breakdown of extracellular adenine nucleotides, such as ATP, to adenosine. Ectonucleotidases include

ectonucleoside triphosphate diphosphohydrolase which can hydrolyze ATP or ADP, ectonucleotide pyrophosphatase/phosphodiesterases, alkaline phosphatases and 5'-nucleotidases (Zimmermann, 2000). When adenosine levels in the extracellular space are high, adenosine is transported into cells by means of transporters. It is then phosphorylated to AMP by adenosine kinase or degraded to inosine by adenosine deaminase. Adenosine deaminase, but not adenosine kinase, is also present in the extracellular space (Fredhoml et al., 2001). Another potential source of extracellular adenosine is cAMP, which can be released from neurons and converted by extracellular phosphodiesterases into AMP and thereafter by an ecto-5'-nucleotidase to adenosine. The transport of adenosine by facilitated diffusion is equilibrative and bidirectional, meaning that the net transport of adenosine either into or out of the cell depends upon the adenosine concentration gradient in both sides of the membrane. Inhibition of adenosine transport can, therefore, inhibit either adenosine release or adenosine uptake, depending upon the intra- and extracellular levels of adenosine (Gu et al., 1995). However, since the extracellular formation of adenosine from released adenine nucleotides constitutes a second source of adenosine, which is not affected by transport inhibition, the transport inhibitors usually cause an increase in the extracellular adenosine levels. Under hypoxic and ischemic conditions there is a marked increase in cytoplasmatic adenosine leading to an intense release of adenosine, which is inhibited by adenosine uptake inhibitors (Parkinson et al., 2002).

Excitatory amino acid-mediated release of adenosine is certainly involved; however, of greater importance is probably the fact that whenever intracellular levels of adenine nucleotides fall as a result of excessive energy use, the intracellular levels of adenosine will rise dramatically (Fredhoml et al., 2001). For example, following hypoxia there is a decrease of intracellular ATP, accompanied by an accumulation of 5'-AMP and subsequently adenosine. The nucleoside is thereafter transported into the extracellular

space via the transporters. Furthermore, when the intracellular level of adenosine is very high, adenosine simply diffuses out of cells. Direct release of intracellular adenine nucleotides, such as ATP, that is thereafter converted extracellularly by ecto-ATPase and ecto-ATP-diphosphohydrolase (ecto-apyrase) to AMP and dephosphorylated by ecto-5'-nucleotidase to adenosine, should also be considered (Zimmermann et al., 2000). Adenosine is neither stored nor released as a classical neurotransmitter since it does not accumulate in synaptic vesicles, being released from the cytoplasm into the extracellular space through a nucleoside transporter. The adenosine transporters also mediate adenosine reuptake, the direction of the transport being dependant upon the concentration gradient at both sides of the membrane (Fredhoml et al., 2001). Since it is not exocytotically released, adenosine behaves as an extracellular signal molecule influencing synaptic transmission without itself being a neurotransmitter, i.e. modulates the activity of the nervous system at cellular level presynaptically by inhibiting or facilitating transmitter release, postsynaptically by hyperpolarising or depolarising neurones and/or exerting non-synaptic effects. Adenosine, therefore, belongs to the group of neuromodulators.

Adenosine receptors

Four adenosine receptor (AR) subtypes (A_1, A_{2A}, A_{2B}, and A_3) have been cloned and pharmacologically characterized, all of which are G protein-coupled receptors (GPCRs). Adenosine receptors can be distinguished according to their preferred mechanism of signal transduction: A_1 and A_3 receptors interact with pertussis toxin-sensitive G proteins of the Gi and Go family; the canonical signaling mechanism of the A_{2A} and of the A_{2B} receptors is stimulation of adenylyl cyclase via Gs proteins. In addition to the

coupling to adenylyl cyclase, all four subtypes may positively couple to phospholipase C via different G protein subunits (Fredholm et al, 2001).

Considering the overall protein structure, ARs display the topology typical of GPCRs. Sequence comparison between the different GPCRs revealed the existence of different receptor families sharing no sequence similarity even if specific fingerprints exist in all GPCR classes. However, all these receptors have in common a central core domain consisting of seven transmembrane helices (TM1-7), with each TM composed of 20–27 amino acids, connected by three intracellular (IL1, IL2, and IL3) and three extracellular (EL1, EL2, and EL3) loops. Two cysteine residues (one in TM3 and one in EL2), which are conserved in most GPCRs, form a disulfide link which is possibly crucial for the packing and for the stabilization of a restricted number of conformations of these seven TMs. Aside from sequence variations, GPCRs differ in the length and function of their N-terminal extracellular domain, their C-terminal intracellular domain, and their intracellular loops. Each of these domains provides very specific properties to these receptor proteins. Particularly, consensus sites for N-linked glycosylation exist on the extracellular regions of ARs, although the precise location of the sites for this post-translational modification varies amongst the AR subtypes. The carboxyl-terminal tails of the A_1AR, $A_{2B}AR$, and A_3AR, but not $A_{2A}AR$, possess a conserved cysteine residue that may putatively serve as a site for receptor palmitoylation and permit the formation of a fourth intracellular loop (Moro et al., 2005).

The A_1AR, $A_{2B}AR$, and A_3AR are very similar in regard to the number of amino acids composing their primary structure, and in general, these AR subtypes are among the smaller members of the GPCR family. For example, the human homologs of the A_1AR, $A_{2B}AR$, and A_3AR consist of 326, 328, and 318 amino acid residues, respectively. Conversely, the human $A_{2A}AR$ is composed of 409 amino acids. It should be noted that the size of ARs deduced from their primary amino acid structure frequently is not

consistent with the mass estimated by polyacrylamide gel electrophoresis of the expressed proteins. The post-translational glycosylation of ARs, which may vary in a cell type-dependent fashion, likely accounts for these discrepancies. The human A_1AR and human A_3AR display 49% overall sequence identity at the amino acid level, while the human $A_{2A}AR$ and human $A_{2B}AR$ are 45% identical (Fredholm et al, 2001).

A_1 adenosine receptors

The A_1 receptor is widely expressed throughout the body, having its highest expression in the brain, spinal cord, atria and adipose tissue (Baraldi et al., 2000). Via adenosine A_1ARs, adenosine reduces heart rate, glomerular filtration rate, and renin release in the kidney; it induces bronchoconstriction and inhibits lipolysis (Elzein and Zablocki, 2008). Adenosine A_1Rs can be coupled to different pertussis toxin-sensitive G proteins, which mediate inhibition of adenylate cyclase and regulate calcium and potassium channels, as well as inositol phosphate metabolism (Fredholm et al., 2001). A_1ARs and $A_{2A}ARs$ are primarily responsible for the central effects of adenosine (Dunwiddie and Masino, 2001). In addition to their postsynaptic locations in different brain regions, A_1ARs can be found presynaptically and modulate neurotransmitter release. Presynaptic A_1ARs are the prototype of GPCRs, the stimulation of which decreases the probability of neurotransmitter release. The main mechanism of A_1AR-mediated inhibition of exocytosis is a direct inhibitory effect on voltage-dependent Ca2+ channels (Moore et al., 2003). $A1_AR$ displays two different affinities for agonist, which have classically been attributed to a different coupling to heterotrimeric G proteins. According to this two independent site model, coupled receptor–G protein complexes display high affinity for agonists and uncoupled receptors display low affinity. The reported cluster-arranged cooperative model predicts that the high- and low-affinity sites are a consequence of the negative cooperativity of agonist binding and do not seem to be related to the content of

G protein-coupled or –uncoupled receptors (Franco et al., 1996). Like other GPCR members, A_1AR expression is regulated in response to agonist or antagonist stimulation. Desensitization of A_1ARs has been described in intact animals and in cell cultures. Prolonged administration of A_1AR agonists to animals leads to functional desensitization of A_1ARs in guinea pig heart, rat adipocytes, rat atrial muscle, and rat brain (Moro et al., 2006). The reduced functional response is attributable to a net loss of A_1ARs or down-regulation, a decrease in the proportion of A_1ARs displaying the high-affinity state for agonists, and a decrease in the content of Gi proteins. The loss of binding sites on the cell membrane owing to internalization of A_1ARs is a slower event. Ser/Thr phosphorylation seems to be related to short-term clustering and desensitization, as well as long-term internalization of A_1ARs (Ciruela et al., 1997).

A_{2A} adenosine receptors

The $A_{2A}AR$ exists in a wide variety of organs including major peripheral tissues (e.g., liver, heart, lung, and the immune system) and the central nervous system (CNS) (Lee et al., 2003). In the developing rat brain, expression of the $A_{2A}AR$ is transiently regulated in various areas (e.g., the striatum, cortex, and hippocampus), perhaps implying a role of adenosine in neuronal development. Soon after neurogenesis, the $A_{2A}AR$ is highly expressed by striatal neurons and co-localizes with the D_2 dopamine receptor in GABAergic striatopallidal neurons (Ferrè et al., 2008). In addition to the intense expression in the striatum, low levels of $A_{2A}AR$ are found in many brain regions (e.g., the cortex and hippocampus) and it has been suggested that adenosine acting at the $A_{2A}AR$ regulates important neuronal functions including neuronal protection and synaptic transmission (Ferrè et al., 2008). Regulation of $A_{2A}AR$ gene expression is therefore likely to play an important role in neuronal development, basal ganglia activity, and many other peripheral functions. In the CNS, l-DOPA enhanced the gene

expression of the striatal $A_{2A}AR$ in 6-OHDA-lesioned rats (Tomiyama et al., 2004). Treatment with an antagonist of the NMDA receptor (memantine) was also reported to elevate the transcript level of striatal $A_{2A}ARs$ (Marvanova and Wong, 2004). The adenosine $A_{2A}AR$ couples primarily to members of the Gs family. Like other GPCRs it can also interact with other G proteins if the receptor is very over-expressed, but the evidence for such coupling in vivo is not compelling. In striatum the $A_{2A}AR$ interacts with Golf proteins (Corvol et al., 2001). It is not known if there are significant differences in receptor affinity or in signaling dependent on which of the two partners (or which variant of Gs) the receptor interacts with. There are instances where other G protein pathways have been implicated, and it will be important to determine if this alternate coupling is a regulated process, for example via phosphorylation. There is no compelling reason to assume that this GPCR coupling to members of the Gs family would signal in anything but a canonical way. Thus, most effects are probably due to activation of adenylyl cyclase and generation of cAMP. The $A_{2A}AR$ can recruit β-arrestin via a GRK-2 dependent mechanism (Khoa et al., 2006). This is influenced by activation of cytokine receptors, which cause reduced desensitization of the $A_{2A}AR$ (Khoa et al., 2006).

One key target of PKA is the cAMP responsive element-binding protein (CREB) which is critical for many forms of neuronal plasticity as well as other neuronal functions (Josselyn and Nguyen, 2005). Phosphorylation of CREB at Ser133 by PKA activates CREB and turns on genes with cAMP responsive elements (CRE sites) in their promoters. One important feature of CREB is that it is a point of convergence for the cAMP/PKA and MAPK pathways. Stimulation of the $A_{2A}ARs$ counteracts the inhibition of neurite outgrowth due to MAPK blockade (Cheng et al., 2002). Stimulation of the $A_{2A}AR$ alone also activates the Ras/Raf-1/MEK/ERK signaling through PKA-dependent and PKA-independent pathways via Src- and Sos- mediated

mechanisms, respectively (Schulte and Fredholm, 2003). Interestingly, phosphorylation/activation of CREB has been shown to compete with nuclear factor-κB (NFκB) p65 for an important co-factor, CBP. Phosphorylated CREB was therefore proposed to mediate the anti-inflammatory effect of the $A_{2A}AR$ receptor and inhibition of NFκB by $A_{2A}AR$ activation during acute inflammation in vivo was demonstrated (Fredholm et al., 2007).

An interesting observation is that activation of $A_{2A}AR$ receptor facilitates activities of adenosine transporters via a PKC-dependent pathway in the hippocampus, and thus reduces the level of extracellular adenosine available for A_1AR activation (Pinto-Duarte et al., 2005). In addition, PKC was shown to play a key role in mediating the enhancement of noradrenaline release by the $A_{2A}AR$ in rat tail artery (Fresco et al., 2004). Activation of multiple signaling pathways by the $A_{2A}AR$ appears to contribute to its diverse and complex functions in various tissues.

A_{2B} adenosine receptors

$A_{2B}AR$ mRNA was originally detected in a limited number of rat tissues by Northern blot analysis, with the highest levels found in cecum, bowel, and bladder, followed by brain, spinal cord, lung, epididymis, vas deferens, and pituitary. The use of more sensitive reverse transcriptase-polymerase chain reaction techniques revealed a ubiquitous distribution of $A_{2B}AR$ (Spicuzza et al., 2006). mRNA encoding $A_{2B}AR$ was detected at various levels in all rat tissues studied, with the highest levels in the proximal colon and lowest in the liver. In situ hybridization of $A_{2B}ARs$ showed widespread and uniform distribution of $A_{2B}AR$ mRNA throughout the brain (Dixon et al., 1996).

Pharmacological identification of $A_{2B}ARs$, based on their low affinity and characteristic order of potency for agonists, also indicates a widespread distribution of $A_{2B}ARs$. In

brain, functional $A_{2B}ARs$ are found in neurons and glial cells. Although there is no evidence that $A_{2B}AR$ are present in microglia, there is ample data that show that they are expressed in astrocytes and in different glioma cell lines (Fiebich et al., 1996). The expression of $A_{2B}ARs$ in glial cells, which represent a majority of the brain cell population, can explain the original observation that slices from all brain areas examined showed an adenosine-stimulated cAMP response.

Functional $A_{2B}ARs$ have been found in fibroblasts and various vascular beds, hematopoietic cells, mast cells, myocardial cells, intestinal epithelial and muscle cells, retinal pigment epithelium, endothelium, and neurosecretory (Gessi et al., 2005). Although activation of adenyl cyclase is arguably an important signaling mechanism for $A_{2A}ARs$, this is not necessarily the case for $A_{2B}ARs$, as other intracellular signaling pathways have been found to be functionally coupled to these receptors in addition to adenyl cyclase. In fact activation of adenosine $A_{2B}ARs$ can increase phospholipase C in human mast cells and in mouse bone marrow-derived mast cells. $A_{2B}AR$ activation also elevates inositol triphosphate (IP3) levels, indicating this receptor can couple also to Gq-proteins. $A_{2B}ARs$ have been implicated in the regulation of mast cell secretion and, gene expression, intestinal function, neurosecretion, vascular tone and in particular asthma (Varani et al., 2005).

A$_3$ adenosine receptors

The A_3AR has widely distributed its mRNA being expressed in testis, lung, kidneys, placenta, heart, brain, spleen, liver, uterus, bladder, jejunum, proximal colon and eye of rat, sheep and humans. However, marked differences exist in expression levels within and among species. In particular rat testis and mast cells express high concentrations of A_3AR mRNA, while low levels have been detected in most other rat tissues (Gessi et al., 2008). Lung and liver have been found as the organs expressing high levels of

A$_3$AR mRNA in human, while low levels have been found in aorta and brain. Lung, spleen, pars tuberalis and pineal gland expressed the highest levels of A$_3$AR mRNA in sheep.

The presence of A$_3$AR protein has been evaluated through radioligand binding, immunoassay or functional assay in a variety of primary cells, tissues and cell lines (Gessi et al., 2008). In the mouse brain a widespread, relatively low level of A$_3$AR binding sites was found (Jacobson et al., 1993). Similar data were obtained in the rat and in gerbil and rabbit brain. Electrophysiological and biochemical evidence suggested the presence of A$_3$ARs in the rat hippocampus and cortex, and functional studies also indicated its presence in the brain. In cardiomyocytes, there was no direct evidence of the presence of A$_3$ARs but several studies reported that it was responsible for cardioprotection in a variety of species and models, including isolated cardiomyocytes and isolated myocardial muscle preparations (Peart and Headrick, 2007). In lung parenchyma and in human lung type 2 alveolar-like cells (A549), the A$_3$AR was detected through radioligand binding and immunohistochemical assays (Varani et al., 2006).

The classical pathways associated with A$_3$AR activation are the inhibition of adenylyl cyclase activity, through the coupling with Gi proteins, and the stimulation of phospholipase C (PLC), inositol triphosphate (IP3) and intracellular calcium (Ca^{2+}), via Gq proteins (Fredholm et al., 2001). However, more recently additional intracellular pathways have been described as relevant for A$_3$AR signaling. For example, in the heart, A$_3$AR mediates cardioprotective effects through ATP-sensitive potassium (KATP) channel activation. Moreover, it is coupled to activation of RhoA and a subsequent stimulation of phospholipase D (PLD), which in turn mediates protection of cardiac myocytes from ischemia (Mozzicato et al., 2004). In addition, in different recombinant and native cell lines, A$_3$AR is involved, like the other adenosine subtypes,

in the modulation of mitogen-activated protein kinase (MAPK) activity (Schulte and Fredholm, 2003). A_3AR signaling in Chinese Hamster Ovary cells transfected with human A_3AR (CHO-hA3) leads to stimulation of extracellular signal-regulated kinases (ERK1/2). In particular, A_3AR signaling to ERK1/2 depends on $\beta\gamma$ release from pertussis toxin (PTX)-sensitive G proteins, phosphoinositide 3-kinase (PI3K), Ras and mitogen-activated protein kinase kinase (Schulte and Fredholm, 2003). It has been reported that A_3AR activation is able to decrease the levels of PKA, a downstream effector of cAMP, and of the phosphorylated form of PKB/Akt in melanoma cells. This implies the deregulation of the Wnt signaling pathway, generally active during embryogenesis and tumorigenesis to increase cell cycle progression and cell proliferation (Fishman et al., 2002). Involvement of the PI3K/PKB pathway has been linked with preconditioning effects induced by A_3AR activation in cardiomyocytes from newborn rats (Germack and Dickenson, 2005). An elegant study has recently documented a role of A_3AR in cell survival signaling in resveratrol preconditioning of the heart. This study provides evidence that resveratrol preconditions the heart through the activation of adenosine A_1 and A_3AR, transmitting a survival signal through both the PI3K-Akt-Bcl2 and, only in the case of A_3AR, cAMP response element-binding protein (CREB)-Bcl2 pathways (Das et al., 2005). Subsequently it has been demonstrated that CREB phosphorylation occurs through both Akt-dependent and -independent signaling. Activation of PI3K-Akt-pBAD by A_3AR has been observed recently in glioblastoma cells leading to cell survival in hypoxic conditions (Merighi et al., 2007). Further studies indicate that A_3AR activation by interfering with PKB/Akt pathways can decrease interleukin-12 (IL-12) production in human monocytes (la Sala et al., 2005). Collectively, these findings demonstrate that several intracellular mechanisms are involved following A_3AR stimulation, the understanding of which may

be essential and crucial for explaining the different aspect of its activation.

Therapeutic potential

Cardiovascular system

A_1AR is the most extensively studied and well characterized of the adenosine receptor subtypes in relation to cardiac protection. The A_1AR is best understood insofar as its effects on injury and in terms of receptor-coupled kinase/protein signaling. For the most part, transduction cascades induced by A_1AR agonism follow those of several other protective GPCR systems (Hausenloy and Yellon, 2004). However, there remain some controversies regarding both signal cascades and A_1AR-mediated responses in the heart. Originally, adenosine (via A_1AR activation) was thought to induce myocardial protection through preservation of ATP (and improved nucleotide repletion on reperfusion), stimulation of glycolysis, and normalization of the hearts so-called "oxygen supply/demand ratio" (Ely and Berne, 1992). Subsequent investigations have identified essential protein kinase signaling cascades together with putative end-effectors (including the mitochondrial KATP channel), in the protective and preconditioning actions of A_1ARs.

Adenosine enhances tolerance to ischemia via metabolic substrate effects (Headrick et al., 2003). Adenosinergic cardioprotection in ischemic-reperfused hearts involves reductions in oncotic and apoptotic death, and improved functional outcomes (Willems et al., 2005). Recent work supports differential effects of acute adenosine vs. transient adenosinergic preconditioning, consistent with multiple pathways of protection (Peart and Headrick, 2003). In terms of cellular targets, adenosine appears to directly protect cardiomyocytes or myocardial tissue (likely via A_1 and A_3ARs), and additionally protects via limiting inflammation and injurious interactions between inflammatory

cells and vascular and myocardial tissue. The different cardioprotective effects of AR agonism have been verified in animal and human tissue (Willems et al., 2005). However, few studies have addressed the possibility that altered AR-mediated protection might underlie specific cardiovascular disorders, though there is evidence to support this. Hypertrophic hearts, for example, display abnormal adenosinergic signaling, and dysregulated adenosine formation. Interestingly, ARs impact on many processes implicated in cardiovascular "aging", regulating Ca^{2+} influx and oxidant injury, substrate metabolism, angiogenesis, myocardial fibrosis, and apoptotic processes (Willems et al., 2005). Given evidence of a role for ARs in intrinsic cardioprotection, mediation of preconditioning, and modifying the above-mentioned processes, alterations in AR signalling could contribute both to ischemic intolerance and emergence of other features of aged myocardium. All ARs are considered to be expressed within cardiovascular cells. Studies in different species verify endogenous adenosine contributes to intrinsic ischemic tolerance, and support cardioprotective roles for A_1ARs in vitro and in vivo, and for $A_{2A}ARs$ in vivo (Willems et al., 2005). Anti-ischemic effects of A_1ARs appear direct (at cardiomyocytes), since similar protection is observed in isolated hearts, cardiomyocytes, and in vivo (Roscoe et al., 2000). Protective $A_{2A}AR$ effects involve modulation of vascular function, platelet adhesion and neutrophil activation. There is currently no direct evidence for acute $A_{2B}AR$ mediated cardioprotection, partially due to lack of selective $A_{2B}AR$ agonists/antagonists.

In contrast to A_1 and $A_{2A}ARs$, there is little evidence that intrinsically activated A_3ARs mediate protection. A_3AR antagonists have no effect on ischemic outcomes in myocytes or hearts (Maddock et al., 2002).

Airways

A role for adenosine in pulmonary disease was first suggested when it was found that adenosine and related synthetic analogues were potent enhancers of IgE-dependent mediator release from isolated rodent mast cells (Holgate et al., 1980). A few years later, adenosine administered by inhalation was shown to be a powerful bronchoconstrictor of asthmatic but, importantly, not of normal airways (Cushley et al., 1983). Further work showed that both allergic and non-allergic asthmatics responded in a similar way and that the effect was also seen with adenosine 5′-monophosphate (AMP), ADP and ATP (Basoglu et al., 2005). Elevated levels of adenosine are present in chronically inflamed airways; they have been observed both in the bronchoalveolar lavage fluid and the exhaled breath condensate of patients with asthma (Caruso et al., 2006). Adenosine levels are also increased after allergen exposure and during exercises in atopic individuals. The observed increase in tissue levels of adenosine suggests that adenosine signaling could regulate important features of chronic inflammatory disorders of the airways, including asthma and chronic obstructive pulmonary disease (COPD). Consistent with the hypothesis of adenosine playing an important role in the pathogenesis of chronic inflammatory disorders of the airways, mice deficient in adenosine deaminase (ADA) develop severe pulmonary inflammation and airway remodeling in association with elevated adenosine concentrations in the lung (Blackburn et al., 2000). The pulmonary phenotype in ADA-deficient mice consists of airway accumulation of eosinophils and activated macrophages, mast cell degranulation, mucus metaplasia in the bronchial airways, and emphysema-like devastation of the lung parenchyma. Although these histological traits do not completely resemble those of human asthma, the ADA-deficient mouse model is a useful tool to study the pathogenic role of adenosine in chronic airway inflammation.

The central role of adenosine in chronic lung inflammation is also supported by studies carried out in mice that have increased levels of interleukin IL-13 in the lung. These mice develop inflammation, fibrosis and alveolar destruction in association with elevated adenosine concentrations in the lung (Caruso et al., 2006). Treatment with ADA to prevent the increase in adenosine concentrations resulted in a marked decrease in the severity of the pulmonary phenotype, suggesting that adenosine mediates IL-13-induced inflammation and tissue remodeling. Blockade of adenosine re-uptake by administration of dipyridamole has been used in humans to test the hypothesis that the accumulation of extracellular adenosine functionally modulates important features of the asthmatic response. In addition, it has been shown that a rapid increase in sputum eosinophilia occurs when asthmatics are exposed to adenosine by means of a provocation test with AMP (van der Berge et al., 2003). Taken together, these observations indicate that adenosine is likely to play an important role in asthma and COPD through interaction with specific cell-surface receptors. Expression of the four identified adenosine receptors has been shown in a large number of proinflammatory and structural cells and recently in the peripheral lung parenchyma of patients with COPD. The affinity of A_1, A_{2A}, and A_3ARs, studied by means of saturation binding assays, was substantially decreased in patients with COPD, whereas their level of expression appears to be increased. Conversely, the affinity of $A_{2B}ARs$ was not altered, but the density was significantly decreased in patients with COPD (Varani et al., 2006). This suggests that adenosine signalling play an important but rather complex role in COPD. Hence, adenosine responses are not only dictated by the bioavailability of the nucleoside but also by the pattern of adenosine receptor expression, which is known to be finely modulated by physiological and/or pathological tissue environments. Stimulation of A_1ARs promotes activation of human neutrophils and enhances neutrophil adhesion to the endothelium in vitro, suggesting a pro-inflammatory role for

this receiver. However, in ADA/A_1ARs double knockout mice, the lack of A_1ARs results in enhanced pulmonary inflammation, mucus metaplasia, alveolar destruction and earlier death from respiratory distress, indicating a protective function. Activation of A_{2A}ARs on activated immune cells by adenosine appears to largely suppress the inflammatory response. In human neutrophils, stimulation of A_{2A}ARs reduces neutrophil adherence to the endothelium, inhibits formyl-Met-Leu-Phe (fMLP)-induced oxidative burst and inhibits superoxide anion generation (Fredholm et al., 1996). In monocytes and macrophages, activation of A_{2A}ARs inhibits lipopolysaccharide-induced tumour necrosis factor-α expression. Therefore, A_{2A}AR agonists might have anti-inflammatory effects in diseases such as COPD, where neutrophil- and monocyte-mediated tissue injury is implicated (Caruso et al., 2006).

Initial evidence for the role of A_{2B}ARs in asthma and COPD came from pharmacological studies of enprofylline, a methylxanthine structurally related to theophylline (Feoktistov and Biaggioni, 1995). It was proposed that the A_{2B}AR might be the therapeutic target in the long-term clinical benefit achieved with relatively low doses of theophylline and enprofylline. Recently, A_{2B}ARs have been shown to mediate several pro-inflammatory effects of adenosine in the large majority of inflammatory and structural cells of the lung. For example, functional human A_{2B}ARs have been identified in mast cells, bronchial smooth muscle cells and lung fibroblasts. In these cells, adenosine, via activation of A_{2B}ARs, increases the release of various inflammatory cytokines, which induce IgE synthesis from human B lymphocytes and promote differentiation of lung fibroblasts into myofibroblasts. Such findings provide support for the view that activation of A_{2B}ARs could enhance the inflammatory response associated with asthma and that selective blockade of these receptors would be potentially beneficial in the treatment of asthma and other pulmonary inflammatory diseases. The functional significance of the A_3AR in the pathogenesis of chronic inflammatory airway

diseases remains controversial largely owing to major species differences (Caruso et al., 2006).

Adenosine and cancer

One of the difficulties in treating most of the common cancers (colon, lung, breast, prostate, etc.) is that they form solid tumors. The individual cancer cells, being different from normal cells, form a tissue mass that behaves in a radically different way from normal tissues in the body. This is because the major cell population (the cancer cells) has grown in a way that is out of step with all of the other cells that would normally form a supportive network. In particular, the growth of the cancer is not coordinated with the development of a proper blood supply. The vascular network of a tumor is usually inadequate, the blood vessels are often too few in number, the network is improperly branched, and their calibre is not well controlled. This means that the blood supply is inadequate. Consequently, most solid tumors do not receive sufficient oxygen and the cells are hypoxic. Specifically, hypoxia is conducive to adenine nucleotide breakdown, which is responsible for the adenosine release (Vaupel et al., 2001). As a consequence, adenosine accumulates to high levels in hypoxic tissues. In particular, it is recognized that significant levels of adenosine are found in the extracellular fluid of solid tumors, suggesting a role of adenosine in tumor growth (Merighi et al., 2003). Adenosine, released from hypoxic tissue, is thought to be an angiogenic factor that links altered cellular metabolism, caused by oxygen deprivation, to compensatory angiogenesis. Angiogenesis (or neovascularization) begins with the migration of endothelial cells, originating from capillaries, into the tissue being vascularized. Adenosine has been reported to stimulate or inhibit the release of angiogenic factors

depending on the cell type examined (Burnstock, 2006). On one hand, adenosine is known to cause the synthesis of vascular endothelial growth factor (VEGF) and increase the proliferation of endothelial cells obtained from the aorta, coronary vessels, and retina (human retinal endothelial cells, HREC). In particular, adenosine has been shown to induce the DNA synthesis in cultures of human umbilical vein endothelial cells (HUVEC) (Burnstock, 2006).

In the human leukemia HL60, human melanoma A375, and human astrocytoma cells, adenosine at millimolar concentrations caused apoptosis. It seems likely that apoptosis is mediated by the intracellular actions of adenosine rather than through surface receptors (Merighi et al., 2002). It has been argued that the effect of high adenosine concentration might be subsequent to uptake of adenosine by the cell and intracellular accumulation of AMP, leading to caspase activation (Merighi et al., 2003).

In many cases, tumor-induced immune suppression is mediated by soluble inhibition factors or cytokines elaborated by the tumor cells. Extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine, suggesting that this autacoid constitutes an important local immunosuppressant within the microenvironment of solid tumors.

Antigen-presenting cells such as dendritic cells and macrophages are specialized to activate naïve T-lymphocytes and initiate primary immune responses. Adenosine inhibits interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α) production in dendritic cells and in macrophages impairing T-cell priming and suppressing the anticancer immune response. Furthermore, adenosine impairs the induction and expansion of cytotoxic T-lymphocytes and the antitumor activity of natural killer (Hoskin et al., 2002).

Differential effects of adenosine on normal and cancer cells have been previously reported, showing that the proliferation of lymphocytes derived from patients with

chronic lymphocytic leukemia was inhibited by adenosine, whereas the proliferation of lymphocytes from healthy people was inhibited to a lesser extent.

In vivo studies have shown that adenosine exerts a profound inhibitory effect on the induction of mouse cytotoxic T-cells, without substantially affecting T-cell viability (Hoskin et al., 2002).

Adenosine sustains a complex role in the immune system activity, because when given to mice pretreated with cyclophosphamide it demonstrated a myeloprotective effect by restoring the number of white blood cells and the percentage of neutrophils as compared with normal values. Furthermore, it has been demonstrated that the elevation of the extracellular adenosine concentrations induced a radioprotective effect in mice by the stimulation of hematopoiesis in the bone marrow and the spleen. In support of this myelostimulatory role, it has been demonstrated that adenosine enhances cycling of the hematopoietic progenitor cells (Pospísil et al., 2001).

The ability of adenosine to specifically inhibit tumor cell growth in vitro and in vivo suggests that the activation and/or blockade of the pathways downstream of adenosine receptors may contribute to tumor development. Furthermore, the extracellular adenosine concentration may be a crucial factor in determining the cell progression pathway, either in the apoptotic or in the cytostatic state (Merighi et al., 2003).

Adenosine and central nervous system

Adenosine levels in the brain extracellular space increase dramatically during metabolically stressful conditions, such as ischemia, seizures, or trauma. Adenosine, acting via its receptors, modulates excitability in the central nervous system (CNS) and has a role in mechanisms of seizure susceptibility, sleep induction, basal ganglia function, pain perception, cerebral blood flow, and respiration (Benarroch, 2008).

Adenosine functions as a natural sleep-promoting agent accumulating during periods of sustained wakefulness and decreasing during sleep. It was suggested that adenosine participates in resetting of the circadian clock by manipulations of behavioural state. Indeed, A_1ARs of the suprachiasmatic nucleus regulate the response of the circadian clock to light (Elliott et al., 2001). The sleep inducing properties of adenosine is in line with its A_1AR-mediated inhibitory action and may involve multiple neuronal populations in the central nervous system; however, the actions upon the basal forebrain nuclei involved in sleep and arousal appear to be particularly important (Ribeiro et al., 2002). In healthy humans, caffeine inhibits psychomotor vigilance deficits from sleep inertia, a ubiquitous phenomenon of cognitive performance impairment, grogginess and tendency to return to sleep immediately after abrupt awakening from intermittent and short sleep periods (van Dongen et al., 2001). It thus emerges that there exists a potential role of adenosine-related compounds and of A_1AR agonists as sleep promoters and adenosine receptor antagonists as arousal stimulators. Adenosine A_1AR agonists have anxiolytic activity in rodent models of anxiety, whereas caffeine and the adenosine A_1AR selective antagonist, cyclopentyltheophylline, have anxiogenic properties. The involvement of adenosine A_1ARs in anxiety was confirmed by the finding that the mice knocked out for this receptor showed increased anxiety-related behaviour. Interestingly, patients suffering from panic disorder, a serious form of anxiety disorder, appear to be particularly sensitive to small amounts of caffeine (Ribeiro et al., 2002). These observations, taken together, suggest that drugs that facilitate adenosine A_1AR-mediated actions may be effective for the treatment of anxiety. Endogenous adenosine, through A_1AR activation, modulates long-term synaptic plasticity phenomena, such as long-term potentiation (LTP), long-term depression (LTD), and depotentiation. In accordance with the notion that synaptic plasticity is the basis for learning and memory in different brain areas, adenosine correspondingly modulates behaviour in various learning and memory

paradigms, and adenosine A_1AR antagonists have been proposed for the treatment of memory disorders (Ribeiro et al., 2002). Cognitive effects of caffeine are mostly due to its ability to antagonise A_1ARs in the hippocampus and cortex, the brain areas mostly involved in cognition, positive actions of caffeine on information processing and performance might also be attributed to improvement of behavioural routines, arousal enhancement and sensorimotor gating (Fredholm et al., 1999).

One of the first pathophysiological roles proposed for adenosine was as an endogenous anti-convulsant (Dragunow et al., 1986). Limitations of the use of adenosine receptor agonists as anti-convulsant drugs are due to their pronounced peripheral side effects as well as central side effects like sedation. To circumvent this limitation, the possibility of using compounds to increase the concentrations of endogenous adenosine was proposed, in particular inhibitors of adenosine kinase (McGaraughty et al., 2001). These compounds would facilitate the increase in extracellular adenosine caused by the seizure, having limited effects in other brain areas where the levels of adenosine are low. The anti-epileptic properties of adenosine are mostly due to the well-known inhibitory actions of A_1ARs upon synaptic transmission in the hippocampus, but not only presynaptic actions upon glutamate release are involved (Khan et al., 2000).

In the spinal cord, adenosine A_1 AR activation produces anti-noceptive properties in acute nociceptive, inflammatory and neuropathic pain tests. Therefore, there was an increasing interest in the development of drugs that, by influencing extracellular adenosine levels, could have analgesic actions. Probably due to the anti-inflammatory actions of adenosine, adenosine kinase inhibitors administered orally are even more effective to reduce inflammatory pain than neuropatic or acute pain (Jarvis et al., 2002). By comparing the anti-nociceptive and anti-inflammatory properties of adenosine kinase inhibitors administered at the ipsilateral or contralateral side of the injury, it was concluded that much of the anti-inflammatory action is locally mediated, whereas the

anti-nociceptive action is systemically mediated, exerted predominantly at the spinal dorsal horn level. Indeed, adenosine kinase inhibitors are able to reduce the increase in *c-fos* expression in the spinal dorsal horn, induced by peripheral injection of an inflammatory (carrageenan) substance (Poon and Sawynok, 1999). Modulation of spinal chord-mediated pain by A_1ARs is probably related to its ability to presynaptically inhibit excitatory transmission to substantia gelatinosa neurones in the spinal cord (Ribeiro et al., 2002).

Anti-depressants are widely used in the treatment of neuropatic pain, but their analgesic efficacy seem to occur irrespective of mood altering effects. Recent studies showed that increases in extracellular adenosine levels and subsequent receptor activation are involved in the peripheral anti-nociceptive effect of amitriptyline in nerve injury-induced neuropatic pain in rats, since co-administration of modest doses of caffeine reduces the action of acutely and chronically administered amitriptyline. Similarly, endogenous adenosine seems to be involved in the anti-allodynic action of amitriptyline in a rat model of painful diabetic neuropathy. Tricyclic anti-depressants, including the active metabolite of amitriptyline, nortriptyline, are potent inhibitors of neuronal uptake of adenosine and this is most probably the mechanism by which amitriptyline interacts with endogenous adenosine. The manipulation of endogenous adenosine by amitriptyline, while important, is unlikely to be the sole mechanism underlying its anti-hyperalgesic action, but the attenuation by modest doses of caffeine (within those easily attained in humans after two cups of strong coffee) raise the possibility that dietary caffeine consumption might influence the efficacy of amitriptyline in alleviating neuropatic pain in humans (Esser and Sawynok, 2000).

Morphine induces the release of adenosine and this also contributes to analgesic action of opioids. In neuropathic rats the release of adenosine induced by morphine is reduced, which might explain a decreased efficacy and potency of opioids in the treatment of

neuropathic pain (Sandner-Kiesling et al., 2001). An increase in the expression of kappa opioid receptors and a decrease in the expression of delta receptors, together with corresponding alterations in delta- and kappa-mediated anti-nociception, were recently detected in mice lacking the $A_{2A}AR$ gene, suggestive of a functional interplay between $A_{2A}AR$ and opioid receptors in the control of pain pathways (Bailey, 2002).

Phase I clinical safety studies in healthy volunteers showed that intrathecal adenosine administration attenuated several types of experimental pain without causing significant side effects. Allosteric modulation of adenosine receptors, namely of A_1ARs, has been attempted with success with the objective of developing drugs that by sinergising with endogenous adenosine action could have minimal side effects in the absence of adenosine. A further advantage of allosteric modulators is that they usually possess some degree of tissue selectivity. Allosteric modulation of adenosine A_1ARs reduces allodynia, and, more interesting, the allosteric modulator T62 was effective not only after intrathecal injection but also after systemic administration, which reinforces the interest of adenosine-related compounds as putative drugs for the treatment of chronic pain associated with hyperalgesia and allodynia (Pan et al., 2001).

A_1AR agonists were conclusively shown to attenuate ischemic or excitotoxic neuronal damage both in vitro (cell cultures, brain slices) and in vivo in different models of ischemia/hypoxia (Wardas, 2002). Using primary cortical or hippocampal cell cultures subjected to hypoxia or glucose deprivation, it was demonstrated that both adenosine and the selective A_1AR agonist CHA reduced the neuronal damage (Lynch et al., 1998). It has been demonstrated that local administration of CADO, an adenosine analogue, can attenuate cell loss in the CA1 region of the rat hippocampus in a model of global forebrain ischemia (temporary occlusion of carotid arteries) (Lynch et al., 1998). Moreover, acute systemic or intracerebroventricular injection of CHA attenuates the

neuronal loss in the hippocampus and improves neurological deficits in gerbils or rats subjected to global forebrain ischemia (Wardas et al., 2002).

Studies in knockout mice or by using pharmacologic blockade indicate that A_{2A}ARs in the striatum modulate locomotor activity. The A_{2A}ARs are highly expressed in medium spiny GABA/enkephalin neurons projecting to the glubus pallidus and may form heterodimers with D_2 dopamine receptors and functionally antagonize the effects of dopamine on striatopallidal neurons (Benarroch 2008). Activation of A_{2A}ARs results in increased GABA release in the globus pallidus, which would lead to disinhibition of the subthalamic nucleus (Ribeiro et al., 2002). The A_{2A}ARs are also coexpressed with A_1ARs in glutamatergic corticostriate terminals and antagonize the presynaptic inhibitory effect of the A_1ARs on glutamate release in the striatum. It has been proposed that increased release of glutamate from the subthalamic nucleus projections to the substantia nigra pars compacta would predispose to excitotoxic injury of these dopaminergic cells whereas presynaptic A_{2A}AR mediated increase in glutamate release from corticostriate synapses may predispose to excitotoxic injury of medium spiny neurons (Bara-Jimenez et al., 2003). Epidemiologic and laboratory data suggest that caffeine may reduce the risk of Parkinson's disease by preventing degeneration of nigrostriatal dopaminergic neurons. Furthermore, caffeine and selective A_{2A}AR antagonists protect against dopaminergic cell loss in several toxin models of Parkinson's disease (Simon et al., 2008). After the first studies demonstrating that the administration of methylxanthines, which have been later recognized as adenosine receptor antagonists, can exert the same behavioural effects as dopamine receptor agonists, the discovery of the colocalization of D_2 and A_{2A}ARs has provided an anatomical basis to the functional antagonism between adenosine and dopamine in the basal ganglia. In particular, A_{2A}ARs and D_2 dopamine receptors colocalize on the dendritic spines of the striatopallidal neurons, playing an important modulatory role on

the excitatory actions of glutamatergic transmission (Hettinger et al., 2001). The final functional result of this interaction is that the activation of the $A_{2A}ARs$ induces hypolocomotion, whereas the opposite is observed after D_2 receptor activation. One explanation of the antagonistic interaction between $A_{2A}AR$ and D_2 receptor stimulation came from the observation that they are both coupled to adenylyl cyclase, but with opposite effects, namely stimulation and inhibition, respectively (Kull et al., 1999). Subsequent studies have provided more in-depth molecular explanations by demonstrating that under physiological conditions, activation of the $A_{2A}ARs$ is responsible for a tonic increase in basal cAMP levels on which dopamine can exert its inhibitory effects (Svenningsson et al., 1999). Furthermore, $A_{2A}ARs$ have been also demonstrated to regulate the phosphorylation status of dopamine- and cAMP-regulated phosphoprotein 32 kDa (DARRP-32), and the PKA/CREB cascade, thereby promoting immediate early genes and enkephalin expression (Svenningsson et al., 2000). More recently, the existence of a physical protein–protein interaction between the two receptor subtypes has been demonstrated, which involves peculiar peptide residues (Canals et al., 2003). This interaction leads to the formation of receptor heteromers in the plasma membrane, which contributes to explain the early observation of agonist affinity loss at the D_2 receptor after activation of the $A_{2A}ARs$. The adenosine/dopamine interaction could have very important implications for basal ganglia functioning. It has provided the molecular and biochemical basis to postulate the possible therapeutic use of $A_{2A}AR$ antagonists in Parkinson's disease, and has prompted chemists to synthesize more selective and clinically useful molecules (Pinna et al., 2005). Besides caffeine and theophylline, which represent the first $A_{2A}AR$ antagonists, the synthetic compounds SCH58261 and ZM241385 have proven extremely potent and selective toward $A_{2A}ARs$, and have represented useful *in vitro* and *in vivo* tools to characterize the pathophysiological role of this receptor subtype in the CNS. Other compounds, such as

KW-6002 (istradefylline) and V2006, are currently undergoing clinical trials as novel approaches in the therapy of Parkinson's disease (Jacobson and Gao, 2006).

POLYGLUTAMINE DISEASES

The group of inherited neurodegenerative diseases designated the polyglutamine diseases share many seminal features with the other families of neurodegenerative diseases (Zoghbi and Orr, 2000). They typically manifest with a late age of onset and, at least during their initial stages, these disorders are characterized by a specific set of clinical signs with pathology limited to a distinct subset of neurons. Like many other neurodegenerative diseases, the polyglutamine diseases have, as a hallmark of pathology, the accumulation of insoluble material within neurons, adding further to the concept of a common pathogenic theme, the generation and accumulation of misfolded proteins.

Nine neurodegenerative diseases have as their disease-causing mutation the expansion of a polyglutamine tract (Table 1). This involves the unstable expansion of a CAG sequence within the coding region of each gene. Thus, these diseases fall within a broader class of disorders, i.e. diseases that involve the expansion of an unstable repetitive element, usually triplet sequences (Gatchel and Zoghbi, 2005). Interestingly, expansions of an unstable nucleotide repeat is a mutational mechanism that appears to be unique to the human genome. Furthermore, although genes that are highly homologous to the polyglutamine genes are present in the genomes of other mammals, the polyglutamine tract is not, suggesting that the polyglutamine stretches are not necessary for normal function.

The polyglutamine disorders include spinobulbar muscular atrophy, Huntington's disease (HD), the spinocerebellar ataxias (SCA1, SCA2, SCA3/MJD, SCA6, SCA7 and SCA17), and dentatorubral-pallidoluysian atrophy (DRPLA). All of these disorders are progressive, typically striking at midlife and having a course that consists of an extended period of neuronal dysfunction followed by neuronal loss and eventually death 10–20 years after onset. Other features that characterize this group of neurodegenerative disorders include a direct relationship between the length of the polyglutamine tract and the severity of the disease, and between the number of glutamines and the age of onset and the severity of the disease (Gatchel and Zoghbi, 2005). Mutant CAG repeats show both germline and somatic instability. Germline instability along with the relationship between repeat length and disease course are the basis for the phenomena of genetic anticipation. As the repeat grows upon passage from generation to generation, affected members in successive generations have an earlier age of onset and more rapidly progressing form of the disease.

The autosomal dominant spinocerebellar ataxias (SCAs) are a complex group of neurodegenerative disorders characterized by progressive cerebellar ataxia of gait and limbs variably associated with ophthalmoplegia, pyramidal and extrapyramidal signs, dementia, pigmentary retinopathy and peripheral neuropathy (Zoghbi et al., 2000). Disease onset is usually between 30 and 50 years of age, although early onset in childhood and onset in later decades after 60 years have been reported. The prognosis is variable depending on the underlying cause of the spinocerebellar ataxia subtype. Epidemiological data indicate that SCAs might be more common than that previously estimated with prevalences of up to 5–7 in 100 000 in some populations (Craig et al., 2004).

The clinical features are caused by a combination of degeneration of the cerebellum, basal ganglia, cerebral cortex, optic nerve, pontomedullary systems, spinal tracts or

peripheral nerves. Although the aetiology of SCAs is still poorly understood, genetic analyses, epidemiological data, neuropathological investigations and new experimental models are providing important new insights into the pathogenic mechanisms. At least 28 distinct loci are responsible for rare Mendelian forms of SCA. Interestingly, a few SCA subtypes, including SCAs 1, 2, 3, 6, 7, 17 and dentatorubral pallidoluysian atrophy (DRPLA), are caused by the expansion of a CAG (DNA sequence coding for glutamine) repeat sequence located within the coding region of specific genes, leading to an abnormally long polyglutamine (polyQ) tract in the encoded proteins named ataxins 1, 2 and 3, alpha 1A-voltage-dependent calcium channel, ataxin 7, TATA box binding protein (TBP) and atrophin 1, respectively. These SCAs show, as common features, the progressive neurodegeneration of neuronal subsets in distinct brain areas and the formation of polyQ-containing protein aggregates forming characteristic nuclear or cytoplasmic inclusions (Zoghbi and Orr, 2000). The age at onset and severity of disease symptoms inversely correlate with the length of the glutamine repeat. A second group of SCAs, including SCAs 8, 10 and 12, are caused by a repeat expansion located outside of the coding region of the disease genes leading to dysregulation of gene expression (Zoghbi and Orr, 2000). While the molecular mechanisms underlying SCAs 8 and 10 are unclear, SCA12 appears to be caused by dysregulation of the activity of the crucial enzyme protein phosphatase 2 (PP2, formerly named PP2A) in cerebellar Purkinje cells. Different mechanisms cause cerebellar ataxia and neurodegeneration in SCAs 5, 13, 14 and 27, where alterations in amino acid composition in beta-III spectrin (SPTBN2) (Ikeda et al., 2006), potassium channel KCNC3 (Waters et al., 2006), protein kinase C (PRKCG) and fibroblast growth factor 14 (FGF14), respectively, elicit disease symptoms in these four SCA subtypes (Ikeda et al., 2006). In the rest of SCAs, the genes and, therefore, the mutations remain to be identified and characterized.

SCA1 is one of several inherited forms autosomal dominant ataxia. Typical of most ataxias, SCA1 consists clinically of gait ataxia dysarthria and bulbar dysfunction, with death usually ocurring between 10 and 15 years after the onset of symptoms. Despite the protein, ataxin-1, being widely expressed in the CNS, the most frequently seen and most severe pathological alterations are restricted to loss of Purkinje cells in the cerebellar cortex, as well as loss of neurons in the inferior olivary nuclei, the cerebellar dentate nuclei and the red nuclei (Mascalchi, 2008).

With the identification of an expanded polyglutamine tract as the mutational basis for several neurodegenerative disorders, a pathogenic mechanism largely dependent on the biochemical property of the polyglutamine tract itself gained quick favor. In contrast, two experiments utilizing SCA1 transgenic mice showed that amino acid residues outside of the polyglutamine had a crucial role in pathogenesis. In the first example an amino acid substitution was made in the nuclear localization signal of ataxin-1, such that the protein could no longer be translocated into the nucleus. When this substitution was placed into a mutant allele of ataxin-1 with an expanded polyglutamine tract that was then used to generate transgenic mice, the mice failed to develop disease (Klement et al., 1998). Thus by restricting the subcellular distribution of mutant ataxin-1 to the cytoplasm of susceptible neurons the protein was no longer pathogenic. Perhaps a more dramatic illustration of the importance of 'host' protein sequence for pathogenesis was shown recently when a site of phosphorylation of ataxin-1 was identified, the serine at position 776 (Emamian et al., 2003). Replacing this serine with an alanine yielded a protein that still was transported to the nucleus, but when transported in a mutant ataxin-1 with 82 glutamines failed to cause disease.

TABLE 1. Characteristics of Polyglutamine diseases

Disease	Gene name	Chromosomal location	Pattern of inheritance	Protein	Normal repeat length	Disease repeat length
Spinobulbar muscular atrophy (Kennedy disease)	*AR*	Xq13–21	X-linked recessive	androgen receptor	9–36	38–62
Huntington disease	*HD*	4p16.3	autosomal dominant	huntingtin	6–35	36–121
Dentatorubral-pallidoluysian atrophy (Haw River syndrome)	*DRPLA*	12p13.31	autosomal dominant	atrophin-1	6–35	49–88
Spinocerebellar ataxia type 1	*SCA1*	6p23	autosomal dominant	ataxin-1	6–44	39–82
Spinocerebellar ataxia type 2	*SCA2*	12q24.1	autosomal dominant	ataxin-2	15–31	36–63
Spinocerebellar ataxia type 3 (Machado-Joseph disease)	*SCA3*	14q32.1	autosomal dominant	ataxin-3	12–40	55–84
Spinocerebellar ataxia type 6	*SCA6*	19p13	autosomal dominant	$\alpha1_A$-voltage-dependent calcium channel subunit	4–18	21–33
Spinocerebellar ataxia type 7	*SCA7*	3p12–13	autosomal dominant	ataxin-7	4–35	37–306
Spinocerebellar ataxia type 17	*SCA17*	6q27	autosomal dominant	TATA binding protein	25–42	45–63

HUNTINGTON'S DISEASE

Huntington's disease (HD) is the most common and well-studied polyglutamine neurodegenerative disorder. It has a prevalence of 3–10 affected subjects per 100.000 individuals in Western Europe and North America (Gil and Rego, 2008). The disorder was first described in the 19th century by George Huntington, who identified both its clinical features and its pattern of familial transmission. However, it was not until 1993 that a multicenter consortium, organized by the Hereditary Disease Foundation, discovered the actual HD gene mutation. This is an unstable expansion of CAG repeats within the coding region of the HD gene, which is located on the short arm of chromosome 4 (4p63) and encodes the protein huntingtin. The mutation results in a stretch of glutamine residues located in the NH2-terminal of huntingtin (The Huntington's Disease Collaborative Research Group, 1993). Although the abnormal protein is ubiquitously expressed throughout the organism, cell degeneration occurs mainly in the brain (Vonsattel and DiFiglia, 1998).

CLINICAL MANIFESTATIONS

Classically described as Huntington's *Chorea* (the Greek word for dance), the first signs of the disease are subtle: clumsiness, difficulties with smooth eye pursuit, and slight uncontrolled and awkward movements. These motor disturbances, associated with the loss of voluntary movement coordination, progress slowly. The involuntary movements of the proximal and distal muscles become more severe and the patients gradually lose their capacity to move and eventually communicate. Bradykinesia and rigidity are also common symptoms in Huntington's patients, especially in late stages of the disease. Death generally occurs as a consequence of heart failure or aspiration pneumonia (Sánchez-Pernaute et al., 2000). In juvenile patients the symptomatology is considerably different, being characterized by bradykinesia, tremors, rigidity and dystonia, and

chorea may be completely absent. Affected children may also suffer epileptic seizures. The majority of patients suffer also from inexplicable muscle wasting and weight loss, despite constant caloric intake (Aziz et al., 2008). Although the cause of these peripheral symptoms is still unclear, a recent study has demonstrated the existence of a positive correlation between the levels of branched chain amino acids (valine, leucine and isoleucine), weight loss and disease progression in HD patients, reinforcing the importance of a systemic metabolic defect in HD pathology (Mochel et al., 2007). A number of endocrine abnormalities have also been reported in HD patients, including increased levels of corticosteroids and reduced levels of testosterone (Markianos et al., 2005). Furthermore, 10–25% of HD patients exhibit diabetes mellitus. The cognitive capacities are also severely affected during the course of HD. The slowing of intellectual processes is the first sign of cognitive impairment in HD patients and deficits in some cognitive functions can in some cases be detected decades before the onset of motor symptoms. These cognitive impairments get worse over time and late-stage HD patients show profound dementia. Manic–depressive behavior and personality changes (irritability, apathy and sexual disturbances) are often part of the psychiatric syndrome. Based on the above features, the criteria used for the diagnosis of HD include: (i) a family history of HD; (ii) progressive motor disability with chorea or rigidity with no other cause; and (iii) psychiatric disturbances with progressive dementia with no other cause (Vonsattel and DiFiglia, 1998). Currently, individuals showing these symptoms are submitted to genetic testing in order to screen for the HD mutation and confirm the diagnosis.

GENETICS

The mutation responsible for HD constitutes a stretch of uninterrupted CAG trinucleotide repeats located near the 5'-end in exon 1 of the *HD* gene coding sequence, which comprises 67 exons. Consequently, mutant huntingtin bears a tract of consecutive glutamine residues in its NH2-terminal, 17 amino acids downstream of the initiator methionine (The Huntington's Disease Collaborative Research Group, 1993). The disease is inherited in an autosomal dominant manner. The normal allele is transmitted from generation to generation in a Mendelian fashion. The mutant allele is unstable during meiosis, changing in length in the majority of intergenerational transmissions, with either slight increases of 1–4 units or decreases of 1–2 units. In rare occasions, larger-sized increases occur in parental transmissions, reflecting a particularly high mutation rate during spermatogenesis (The Huntington's Disease Collaborative Research Group, 1993). The number of CAG repeats (i.e. glutamine residues) is the primary and major determinant of disease severity, accounting for 30–60% of the variance in the age of onset of the first symptoms. The remaining variance is attributable to other genetic features and environmental factors (Arning et al., 2005). Interestingly, in a recent study that involved 167 HD patients, the length of the polyglutamine tail (which ranged from 41 to 45 units) accounted for only 30.8% of the variance in the age of onset, whereas 12.3% additional variance could be attributed to genotype variation in the gene coding for the *N*-methyl-D-aspartate (NMDA) receptor subunit NR2B and 4.5% to genotype variation in the gene coding for the NMDA receptor subunit NR2A (Arning et al., 2005). Normally, asymptomatic individuals have >35 CAG repeats whereas HD is manifested when the number of repeats exceeds this threshold. Alleles with 35–39 repeats are associated with later onset of the disease, although incomplete penetrance has been observed in some individuals who have shown no symptoms or neuropathological signs. Alleles of 40–50 units give rise to the most

common adult-onset form of the disease, whereas the longest repeats (normally associated with high allele instability during parental transmission) are responsible for the severe juvenile and infantile cases (The Huntington's Disease Collaborative Research Group, 1993). Importantly, there is a positive correlation between the number of CAG repeats and the Vonsattel grades of neuropathological severity, with greater CAG repeat lengths being associated with greater degrees of cell death in the striatum and higher Vonsattel grades (Vonsattel and DiFiglia, 1998).

PATHOGENESIS

Huntingtin is a protein composed of >3100 amino acids and with a molecular mass of ca 349 kDa, depending on the exact number of glutamine residues. A polymorphic proline-rich segment follows the polyglutamine tail (The Huntington's Disease Collaborative Research Group, 1993). Huntingtin does not show structural homology with any other known protein. The wild-type protein is widely expressed throughout the body, in both neuronal and non-neuronal cells, raising the questions of how and why the disease mutation results in a selective neuronal loss. In the HD brain, particularly in the two most affected regions, the striatum and cerebral cortex, an altered intracellular localization and perinuclear accumulation of mutant huntingtin is observed, with the formation of neuronal intranuclear inclusions (NIIs) and aggregates in dystrophic neurites. However, the neurons bearing inclusions do not correspond to the most vulnerable ones. Indeed, some interneurons that are spared during the course of the disease are the ones that display the highest frequency of aggregates, raising the question of whether inclusions are toxic, protective, or just an epiphenomenon of the disease mechanism.

Functions of wild-type huntingtin

Pioneer studies have suggested that wild-type huntingtin is essential for normal embryonic development, as engineered knockout mutations that disrupt exon 4, exon 5, or the promoter of the mouse HD gene homolog *Hdh*, leading to its complete inactivation, and result in embryonic lethality (Zeitlin et al., 1995). However, a subsequent study has shown that mutant human huntingtin can compensate for the absence of endogenous huntingtin, rescuing the embryonic lethality of mice homozygous for a targeted disruption of the endogenous *Hdh* gene (Leavitt et al., 2001). Thus, it is reasonable to suggest that huntingtin's function during embryonic development is independent of the length of the polyglutamine tail. Moreover, the HD mutation does not seem to abrogate the developmental functions of huntingtin, as HD patients appear to develop normally and the symptoms only start to manifest several years after birth. Furthermore, some evidence suggests that huntingtin might be required not only during embryogenesis but also throughout life, as conditional knockout mice in which the *Hdh* gene is inactivated during adulthood are sterile, develop a progressive motor phenotype and show a reduced life span (Dragatsis et al., 2000). Nevertheless, these studies have not been replicated and, thus, the actual role of normal huntingtin during adulthood is still under debate.

Within the cell, wild-type huntingtin is mainly localized in the cytoplasm associated with organelles such as mitochondria, the Golgi apparatus, the endoplasmic reticulum, synaptic vesicles and several components of the cytoskeleton. Wild-type huntingtin is also present inside the nucleus, although to a lesser extent (Landles and Bates, 2004).

Yeast two-hybrid screenings, affinity pull-down assays, Western blotting and immunoprecipitation studies have shown that wild-type huntingtin binds to numerous proteins. Accordingly, a recent study has identified 234 high-confidence huntingtin-associated proteins (Kaltenbach et al., 2007). Relevant examples include: (i) huntingtin-

associated protein 1 (HAP1), a novel protein with at least two isoforms (HAP1-A and HAP1-B), which is expressed in several tissues including the brain and which interacts with the p150 subunit of dynactin, thus being involved in intracellular transport; (ii) huntingtin-interacting protein 1 (HIP1), a protein that binds to α–adaptin and clathrin, and is thus implicated in cytoskeleton assembly and in endocytosis; (iii) huntingtin-interacting protein 2 (HIP2), a ubiquitin-conjugating enzyme that catalyzes the covalent attachment of ubiquitin units to intracellular proteins, tagging them for degradation by the proteasome; (iv) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme; and (v) microtubules and β-tubulin, which are components of the cytoskeleton (Li and Li, 2004). As expected, the presence of an expanded polyglutamine tail can disrupt and/or modify the nature of the interactions between huntingtin and these interacting proteins, ultimately inhibiting and/or modifying the normal function of these proteins.

Wild-type huntingtin promotes the expression of brain-derived neurotrophic factor (BDNF) by interacting with the transcriptional factor complex repressor-element-1 transcription factor (REST)–neuron-restrictive silencer factor (NRSF) in the cytoplasm and preventing this complex from translocating into the nucleus and binding to the neuron-restrictive silencer element (NRSE) present in the promoter of the BDNF gene (Zuccato et al., 2003). Wild-type huntingtin also promotes the vesicular transport of BDNF along the microtubules through a mechanism that involves HAP1 and the p150 subunit of dynactin. The interaction of wild-type huntingtin with both HAP1 and mixed-lineage kinase 2 (MLK2) has been shown to promote the expression of NeuroD, a helix–loop–helix transcription factor that is crucial for the development of the dentate gyrus of the hippocampus and for the morphogenesis of pancreatic islets (Huang et al., 2002). Wild-type huntingtin prevents the activation of caspase-8 by interacting with HIP1 and preventing the formation of the caspase-activating complex HIP1–HIP1-

protein interactor. Furthermore, neurons from HIP1-knockout mice were recently shown to be less sensitive to NMDA-induced cell death than wild-type neurons, suggesting that HIP1 may play an important role during excitotoxicity (Metzler et al., 2007).

Importantly, wild-type huntingtin is believed to have a pro-survival role in the cell. The antiapoptotic function of wild-type huntingtin has been corroborated in several *in vitro* studies. These have demonstrated that expression of the full-length protein protected conditionally immortalized striatal-derived cells from a variety of apoptotic stimuli. Wild-type huntingtin appeared to act downstream of mitochondrial cytochrome c release, preventing the formation of a functional apoptosome complex and the consequent activation of caspase-9 and caspase-3 (Rigamonti et al., 2001). Moreover, it was recently shown that wild-type huntingtin physically interacts with active caspase-3 inhibiting its proteolytic activity (Zhang et al., 2006). On the other hand, *in vivo* overexpression of full-length wild-type huntingtin bearing 18 glutamine residues in yeast artificial chromosome (YAC)18 transgenic mice conferred significant protection against apoptosis triggered by NMDA receptor-induced excitotoxicity.

Interestingly, the huntingtin sequence contains several HEAT (huntingtin, elongation factor 3, protein phosphatase 2A, target of rapamycin 1) repeats, which are conserved repeating segments of 40 amino acids that form two hydrophobic α-helices. According to a model proposed on a recent study, full-length wild-type human huntingtin may be entirely composed of HEAT repeats stacking on top of each other to form an elongated superhelix (Li et al., 2006). The function of these motifs is still unclear, although they are found in a variety of proteins that are involved in intracellular transport and chromosomal segregation. Furthermore, the 18 amino acids of the huntingtin N-terminus constitute an amphipathic α-helical membrane-binding domain that can reversibly target the protein to the endoplasmic reticulum. As this association is affected by endoplasmic reticulum stress, it was recently suggested that wild-type huntingtin

may function as an endoplasmic reticulum-associated protein that can translocate to the nucleus and back out in response to endoplasmic reticulum stress (Gil and Rego, 2008). Overall, these studies indicate that wild-type huntingtin may exert a variety of intracellular functions such as: (i) protein trafficking; (ii) vesicle transport and anchoring to the cytoskeleton; (iii) clathrin-mediated endocytosis; (iv) postsynaptic signaling; (v) transcriptional regulation; and (vi) anti-apoptotic function.

Aggregation of mutant huntingtin

The normal tertiary protein conformation is destabilized by the presence of the expanded polyglutamine tract, leading to the establishment of abnormal protein–protein interactions with other polyglutamine-bearing proteins (including other molecules of mutant and wild-type huntingtin). This results in the formation of insoluble β-pleated sheets that form polar zipper structures via hydrogen bonding (Stott et al., 1995).

Transglutaminases are enzymes that are involved in the cross-linking of glutamine residues and thus may also participate in the formation of aggregates. Indeed, *in vitro* studies have shown that huntingtin is a transglutaminase substrate and that the transglutaminase-mediated crosslinking increases with the length of the polyglutamine stretch. Hence, the expanded polyglutamine tract caused by the disease mutation could result in an increased transglutaminase-mediated crosslinking with other molecules of mutant and wild-type huntingtin, as well as other polyglutamine-containing proteins, leading to the precipitation and intraneuronal accumulation of protein complexes. In support of this model, transglutaminase activity has been shown to be increased in HD brains (Gil and Rego, 2008).

Some evidence suggests that, once formed, these aggregates of mutant huntingtin may be transported by the microtubule organization center, or centrosome, to a perinuclear localization. However, these results have not been recently replicated and the co-

localization of the aggregates of mutant huntingtin with the centrosome is still under debate.

Because the formation of mutant huntingtin aggregates is regarded as a hallmark of HD, many authors hypothesize that aggregation of mutant huntingtin is the trigger point that eventually leads to cell demise in HD. Several proteins have been shown to abnormally interact and be recruited into the aggregates of mutant huntingtin, causing a severe deregulation of several key intracellular pathways. Nevertheless, mutant huntingtin inclusions may only represent a side-effect of the ongoing cell dysfunction, or may even exert a protective role during the early stages of the disease (Kuemmerle et al., 1999). Indeed, it is possible that the inclusions represent a means for the cell to sequester the toxic N-terminal fragments and oligomers of mutant huntingtin as well as other misfolded proteins which in the soluble form could cause more rapid and severe damage (Gil and Rego, 2008). In line with this hypothesis, *in vitro* studies have demonstrated that the presence of NIIs does not correlate with huntingtin-induced death. Indeed, exposure of striatal neurons transfected with mutant huntingtin to conditions that suppress the formation of NIIs resulted in increased neuronal while the formation of NIIs leads to a decrease in the levels of diffuse mutant huntingtin and to an increase in neuronal survival (Arrasate et al., 2004).

Huntingtin post-translational modifications

Importantly, the activity of both wild-type and mutant huntingtin can be modulated by several post-translational modifications. Within this scenario, several studies have indicated that huntingtin phosphorylation at various sites is neuroprotective through preventing cleavage of mutant huntingtin and its consequent aggregation. Thus, huntingtin can be phosphorylated on serine 421 by protein kinase B or Akt and the closely related serum- and glucocorticoid-inducible kinase, and this attenuates mutant

huntingtin toxicity *in vitro* (Gil and Rego, 2008). Moreover, endogenous phosphorylation of mutant huntingtin on serine 421 is significantly reduced in the striatum of YAC-transgenic mice. Huntingtin can also be phosphorylated at serine 434 by the cyclin-dependent kinase 5, and this phosphorylation reduces caspase-mediated huntingtin cleavage at residue 513 and attenuates aggregate formation and toxicity *in vitro*. Finally, in a recent study, the phosphorylation sites of full-length huntingtin were mapped by mass spectrometry. More than six novel serine phosphorylation sites were identified and mutation of one of these sites, which lies in the proteolytic susceptibility domain (serine 536), inhibited calpain-mediated cleavage and reduced toxicity of mutant huntingtin (Schilling et al., 2006).

Other huntingtin post-translational modifications have also been described. Among them, the reversible binding of a small ubiquitin-related protein modifier (SUMOylation), which is thought to alter inter- and/or intra-molecular interactions of the modified substrate and thus modulate its subcellular localization, activity and stability, was shown to occur in a *Drosophila* model of HD. In this model, the SUMOylation of a pathogenic fragment of huntingtin on lysine residues exacerbated neurodegeneration, whereas mutation of these lysines prevented SUMOylation of the huntingtin fragment and reduced HD pathology in this model (Steffan et al., 2004). On the other hand, palmitoylation (i.e. the post-translational addition of palmitic acid) is believed to play a role in intracellular trafficking (including endocytosis, protein recycling and transport) and the association of cytosolic proteins with membranes have recently demonstrated that normal huntingtin is palmitoylated at cysteine 214 by the palmitoyl transferase huntingtin-interacting protein 14 (HIP14), and this post-translational modification is essential for its trafficking and function. Moreover, the HD mutation reduces the interaction between huntingtin and HIP14, resulting in a marked reduction in huntingtin palmitoylation. As the mutation of the palmitoylation site of

huntingtin and the down-regulation of HIP14 increases inclusion formation *in vitro*, it has been suggested that inhibition of palmitoylation might contribute to NII formation (Yanai et al., 2006).

Huntingtin toxic gain of function

The expanded polyglutamine is believed to confer a new function to huntingtin that is toxic to the cell (toxic gain of function). Indeed, the mutant protein (in either its soluble or its insoluble aggregate form) has been shown to disrupt several intracellular pathways by abnormally interacting and/or sequestering key components of these multiple pathways into the aggregates. On the other hand, several lines of evidence also suggest that a loss of function of wild-type huntingtin (due to its decreased expression and/or sequestration into the aggregates by interacting with the mutant protein) also contributes to the disruption of intracellular homeostasis, culminating in neuronal dysfunction and death (Gil and Rego, 2008).

Protease cleavage sites for caspase-3 and calpain have been identified within the first 550 amino acids of huntingtin, and proteolysis has been shown to increase in the presence of longer polyglutamine tails (Sun et al., 2002). It has been suggested that caspase-3- and calpain-mediated sequential proteolysis of mutant huntingtin produce N-terminal fragments that are more toxic and prone to aggregate and that diffuse passively into the nucleus due to its smaller size (Sun et al., 2002). These fragments can in turn recruit more proteases into the aggregates, favoring their subsequent activation. This creates a positive feedback loop that results in further aggregation and proteolytic cleavage that may ultimately contribute to cell death.

Furthermore, the expanded polyglutamine also reduces the ability of huntingtin to bind and inhibit active caspase-3 and to bind HIP1, which is then free to associate with

HIPPI and induce the activation of caspase-8, an initiator caspase that triggers the apoptotic cascade (Gil and Rego, 2008).

Heat-shock proteins (HSPs) such as HSP40 and HSP70, which are chaperones involved in the refolding of misfolded proteins, are also sequestered into aggregates of mutant huntingtin *in vitro* (Wyttenbach *et al.*, 2000). As the abnormally long polyglutamine tract is likely to result in an overall misfolding of huntingtin, the interaction of HSPs with mutant huntingtin may represent an attempt of the cell to refold the mutant protein. In line with this idea, it has been shown that Hsp70 and Hsp40 interfere with the aggregation of mutant huntingtin *in vitro*, preventing the aggregate-induced inhibition of transcription factors such as the thymidine–adenine–thymidine–adenine (TATA)-binding protein (TBP). However, by being sequestered into the aggregates, these chaperones will eventually be prevented from exerting their normal protective functions and, over the years, this is likely to lead to an intracellular accumulation of misfolded proteins.

Furthermore, several components of the proteasome, such as its regulatory and catalytic subunits and ubiquitin conjugation enzymes, are also sequestered in these aggregates *in vitro* and *in vivo* resulting in the impairment of the ubiquitin–proteasome system (Bence et al., 2001). On the other hand, the presence of ubiquitin residues in mutant huntingtin aggregates may be the result of an unsuccessful attempt of the cell to tag the mutated protein for the proteasome. However, the HD mutation may render the protein resistant to proteasomal degradation. Indeed, the mutant protein with its expanded polyglutamine tail may physically block the proteasome, preventing the entrance of further substrates into the proteasome complex.

Importantly, studies using a conditional HD mouse model (in which silencing of mutant huntingtin expression leads to the disappearance of intranuclear aggregates) showed that aggregate formation is a balance between the rate of huntingtin synthesis and its

degradation by the proteasome (Martin-Aparicio et al., 2001). Therefore, over the course of the disease the proteasome degradation system may become overloaded with an increasing number of misfolded and mutated proteins in the cell. As a consequence, the neurons may be progressively depleted of functional proteasomes, which will lead to a progressive accumulation of misfolded and abnormal proteins, further increasing the rate of protein aggregation. Finally, an impairment of the ubiquitin–proteasome system can induce cell death either by apoptosis or by autophagy (a process by which lysosomes degrade cytoplasmic proteins and organelles), causing the recruitment of lysosomes and proteins involved in the formation of autophagic vacuoles into inclusion bodies (Iwata et al., 2005).

Numerous transcription factors have been reported to interact with mutant huntingtin. Examples include CREB-binding protein (CBP), specific protein-1 (Sp1), and the TBP-associated factor $(TAF)_{II}130$, all of which directly interact with mutant huntingtin through the expanded polyglutamine tail. Other nuclear proteins, such as the pro-apoptotic transcription factor p53, also interact with mutant huntingtin via its SH3 sequences, which are distal to the polyglutamine tract (Landles and Bates, 2004).

Under normal conditions, CBP (a co-activator of the CRE-mediated transcription pathway) is recruited upon phosphorylation, acting as a bridge between CREB and the basal transcriptional machinery. Moreover, CBP can also play a role in histone acetylation by acting as an acetyltransferase, which opens the chromatin structure and exposes the DNA to transcription factors. In the presence of mutant huntingtin, CBP is depleted and/or recruited into huntingtin aggregates, leading to histone hypoacetylation and inhibition of CBP-mediated transcription (Jiang et al., 2006). Furthermore, CRE-mediated transcription is also regulated by $TAF_{II}130$, which is part of the basal transcriptional machinery and can abnormally interact with mutant huntingtin, rendering the transcriptional complex ineffective (Dunah et al., 2002).

Sp1 is a regulatory protein that binds to guanine–cytosine boxes and mediates transcription through its glutamine-rich activation domains which target components of the basal transcriptional complex, such as $TAF_{II}130$. Sequestration of Sp1 and $TAF_{II}130$ into NIIs leads to the inhibition of Sp1-mediated transcription (Dunah et al., 2002). Furthermore, it has also been shown that, despite normal protein levels and nuclear binding activity, the binding of Sp1 to specific promoters of susceptible genes is significantly decreased in transgenic HD mouse brains, striatal HD cells and human HD brains, suggesting that mutant huntingtin dissociates Sp1 from target promoters, inhibiting the transcription of specific genes (Chen-Plotkin et al., 2006). Furthermore, it was also shown that shorter N-terminal huntingtin fragments, which are more prone to misfold and aggregate, are more competent to bind and inhibit Sp1. Interestingly, this effect was reversed *in vitro* by HSP40, a molecular chaperone that reduces the misfolding of mutant huntingtin (Cornett et al., 2006).

On the other hand, mutant huntingtin may also lose the ability to bind and interact with other transcription factors regulated by wild-type huntingtin, as is the case of the NRSE-binding transcription factors. In this particular case, the failure of mutant huntingtin to interact with REST/NRSF in the cytoplasm leads to its nuclear accumulation, where it binds to NRSE sequences and promotes histone deacetylation, leading to the remodeling of the chromatin into a closed structure. As a result, there is a suppression of NRSE-containing genes, including the BDNF gene (Zuccato et al., 2003). This is one case where the loss of the normal huntingtin function can have profound effects, leading to decreased levels of BDNF, an important survival factor for striatal neurons. Indeed, BDNF-knockout models were recently shown to largely recapitulate the expression profiling of human HD, suggesting that striatal medium-sized spiny neurons suffer similar insults in HD and BDNF-deprived environments.

More recently, expression of mutant huntingtin has been shown to down-regulate the expression of the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) Weydt *et al.*, 2006), a transcriptional coactivator that regulates several metabolic processes, including mitochondrial biogenesis and respiration, processes that have been shown to be deregulated in both animal models and patients with HD. Interestingly, decreased expression of PGC-1α was observed in medium-sized spiny neurons, largely affected in HD, whereas striatal interneurons showed increased mRNA levels for PGC-1α which could, at least partially, explain the different vulnerability of these striatal neuronal populations (Weydt et al., 2006).

The formation of neuropil aggregates of mutant huntingtin has led to the hypothesis that axonal transport may be impaired in HD. Indeed, it is believed that normal huntingtin can play a role during axonal transport, perhaps via association with HAP1, promoting both retrograde and anterograde transport. The expanded polyglutamine tract may inhibit this function, compromising the bidirectional transport of different cargoes along the axons, further implicating the loss of wild-type huntingtin function in HD (Gunawardena and Goldstein, 2005). In fact, mutant huntingtin appears to be responsible for altering the wild-type huntingtin/HAP1/p150 complex, causing an impaired association between motor proteins and microtubules, and attenuating BDNF transport, which results in loss of neurotrophic support.

Alternatively, aggregates may physically block transport within narrow axonal terminals (Gunawardena and Goldstein, 2005). Indeed, dystrophic striatal and corticostriatal neurites in HD brains exhibit several characteristics of blocked axons, such as accumulation of vesicles and organelles in swollen axonal projections and multiple huntingtin aggregates. Interestingly, the appearance of striatal axonal inclusions are better correlated with striatal neuronal loss than the formation of NIIs (Gunawardena and Goldstein, 2005).

A direct consequence of impaired axonal transport is disruption of neuronal synaptic transmission. Specifically, this may occur by the depletion of synaptic vesicles and proteins involved in vesicle recycling and receptor endocytosis from the nerve terminals.

Moreover, a number of studies suggest that mutant huntingtin can lead to synaptic dysfunction by altering the availability of various synaptic proteins (Smith et al., 2005). Examples include a progressive depletion of complexin II, a protein that regulates the fusion of synaptic vesicles with the presynaptic plasma membrane and a decrease in the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein synaptobrevin-2, and the G protein rabphilin 3A in the HD striatum. A depletion of PACSIN 1/syndapin (a neurospecific phosphoprotein involved in receptor recycling) in synapses, due to its interaction with the mutant huntingtin SH3 domains, has been also demonstrated. These effects may result from a direct abnormal interaction between the mutant protein and some of the synaptic protein, which may lead to their sequestration in huntingtin inclusions. Alternatively, they may represent a consequence of the altered gene transcription, which may cause the down-regulation of some of these synaptic proteins.

Furthermore, wild-type huntingtin interacts with various vesicle proteins that are important for endocytosis. These interactions may be impaired in the presence of the mutation, thus compromising the synaptic process, which emphasizes the importance of the loss of wild-type huntingtin function (Li et al., 2003).

Mutant huntingtin can also induce synaptic dysfunction by disrupting the expression and activity of several pre- and postsynaptic neurotransmitter receptors. In agreement, it was found that some Huntington' disease mice model (R6/2) striatal neurons contain constitutively abnormal NMDA receptors that display increased responses to NMDA and a decreased Mg^{2+} sensitivity (Starling et al., 2005). These changes were detected as

early as 2 weeks of age. On the other hand, a significant decrease in the percentage of R6/2 striatal neurons expressing the NMDA subunit NR2A was also detected at an early stage. This decrease was not associated with any significant change in the expression of NR1 or NR2B and seemed to be more pronounced in enkephalin-containing neurons. Furthermore, the mRNA and protein levels of the metabotropic glutamate receptors (mGluR)1, 2 and 3 were found to be reduced in R6/2 mice. Importantly, mGluR2 is a presynaptic receptor that regulates glutamate release. Therefore, a reduction in this receptor can lead to an increased release of glutamate due to a decreased feedback control, thus contributing to excitotoxicity.

Mechanisms of neurodegeneration

Over the course of HD, intracellular dysfunction induced by mutant huntingtin progressively leads to the degeneration of important neuronal pathways and cell loss in the striatum, select layers of the cerebral cortex, and other brain regions. Although not necessarily a direct result of the mutant protein, various mechanisms such as excitotoxicity, dopamine toxicity, metabolic impairment, mitochondrial dysfunction, oxidative stress, apoptosis and autophagy have been implicated in HD pathology. Many of these mechanisms may slowly develop over time, becoming increasingly pronounced by the late stages of the disease. Moreover, they are not mutually exclusive and are likely to occur in parallel and promote each other, ultimately culminating in neuronal loss (Gil and Rego, 2008).

Pioneering studies have shown that intrastriatal injections of the NMDA agonist quinolinic acid (QA) induce selective neuronal loss both in rats and in primates resembling the pattern of striatal cell death observed in the human HD condition (Ferrante et al., 1993). However, these results have been disputed and others have shown that QA-induced striatal cell death does not spare medium-sized aspiny neurons,

as is the case in the human HD brain. With the generation of transgenic rodent models of the disease, a unique opportunity was established to assess the influence of expanded polyglutamine tracts on the susceptibility of striatal neurons to excitotoxicity *in vivo*. However, results ranging from an increased susceptibility to an excitotoxic insult to a complete resistance to excitotoxicity were reported (Morton and Leavens, 2000). This discrepancy seams to be related with the different transgenic models used in different studies and particularly with the size of the transgene that they express. Thus, transgenic mice expressing a huntingtin fragment that corresponds to 3% of the total gene (the R6/1 and R6/2 mice) are resistant to KA- or QA-induced excitotoxicity and transgenic rodents expressing 20–30% of the full-length mutant huntingtin respond normally to such insults, whereas transgenic mice expressing the full-length gene (the YAC mice) show an increased susceptibility to excitotoxicity (Gil and Rego, 2008). Although the mechanism responsible for the observed resistance of R6/1 and R6/2 mice to excitotoxic insults is not fully understood, it does not appear to be the result of a reduction in the number of NMDA receptors or a decreased receptor-mediated influx of Ca^{2+}. It is possible that a reduction in the excitatory input to the striatum observed in older R6/2 mice could contribute to this resistance. Moreover, the frequency of spontaneous GABAergic synaptic currents and the expression of the ubiquitous α1 subunit of GABA(A) receptors were shown to be significantly increased in medium-sized spiny projection neurons from symptomatic R6/2 and R6/1 mice (Cepeda et al., 2004). These alterations in inhibitory synaptic input can potentially provide a neuroprotective mechanism to counteract excitotoxicity in these transgenic mice. Alternatively, the resistance phenomenon may be similar to pre-conditioning, as observed with ischemia, where permanent exposure to low levels of excitotoxicity and to changes in intracellular Ca^{2+} homeostasis allows neurons to adapt to an even greater excitotoxic insult and sudden increase in intracellular Ca^{2+} concentration. Accordingly, while corticostriatal

brain slices from pre-symptomatic R6/2 mice showed an increased sensitivity to an ischemic insult (oxygen and glucose deprivation), slices derived from symptomatic and late-stage R6/2 mice were shown to be resistant to ischemia (Klapstein and Levine, 2005).

Despite these controversial results, the striatum receives excitatory glutamatergic inputs from the entire cerebral cortex and the selective vulnerability displayed by striatal neurons in HD may be due to the vast glutamatergic inputs they receive and/or the particular types of glutamate receptors expressed in these cells. Thus, striatal interneurons, which are less affected in HD, have fewer excitatory inputs than striatal projection neurons. Moreover, most medium-sized spiny GABAergic projection neurons express high levels of the NMDA receptor NR2B subunit, which increases the receptor channel permeability and determines its sensitivity to glycine and to Mg^{2+} blockage, and the channel deactivation time. Correspondingly, the spared interneurons express much lower levels of this subunit, which probably confers on them a higher threshold for activation (Sieradzan and Mann, 2001). In agreement with this hypothesis, *in vitro* studies have shown that coexpression of mutant huntingtin and the NMDA receptor subunits NR1A and NR2B enhances excitotoxic cell death with apoptotic features. Furthermore, polyglutamine expansion interferes with the ability of wild-type huntingtin to interact with PSD-95, resulting in the sensitization of NMDA receptors and promoting glutamate-mediated excitotoxicity (Sun et al., 2001). Mutant huntingtin can also increase tyrosine phosphorylation of NMDA receptors, further promoting their sensitization. Finally, mGluR5 is greatly expressed in the postsynaptic membranes of striatal projection neurons. The activation of these facilitatory receptors enhances the responses of NMDA receptors to glutamate activation, contributing to excitotoxicity (Vonsattel and DiFiglia, 1998). The involvement of mGluR5 and NR2B glutamate

receptors in mutant huntingtin-induced excitotoxicity and cell death has also been confirmed in striatal medium spiny neurons from YAC mice (Tang et al., 2003).

Another factor that can contribute to the vulnerability of striatal neurons to excitotoxicity is the capacity of the surrounding glial cells to remove extracellular glutamate from the synaptic cleft. In agreement, a decrease in the mRNA levels of the major astroglial glutamate transporter (GLT1) and the enzyme glutamine synthetase were detected in the striatum and cortex of R6/1 and R6/2 mice. These changes in expression were accompanied by a concomitant decrease in glutamate uptake and could be detected before any other evidence of neurodegeneration, suggesting that a defect in astrocytic glutamate uptake is an early event in HD (Liévens et al., 2001). Furthermore, it was recently reported that mutant huntingtin accumulates in the nucleus of glial cells in HD brains, decreasing the expression of the glutamate transporter. Moreover, while wild-type glial cells protected neurons against mutant huntingtin-mediated neurotoxicity, glial cells expressing mutant huntingtin increased mutant huntingtin-induced neuronal vulnerability, suggesting that a decrease in glutamate uptake by glial cells may indeed exacerbate neuronal excitotoxicity in HD (Shin et al., 2005).

It is also important to note that activation of pathways that lead to the production of excitotoxins in the brain is likely to have an impact in HD. Indeed, recent studies have reported an increase in the endogenous levels of the NMDA-receptor agonist QA (a product of tryptophan degradation generated along the kynurenine pathway) and of its bioprecursor, the free radical generator 3-hydroxykynurenine (3-HK) in the striatum and cortex of early stage HD patients. 3-HK levels were also significantly and selectively elevated in the striatum, cortex and cerebellum of R6/2 mice starting at 4 weeks of age, while both 3-HK and QA levels were increased in the striatum and cortex of full-length HD mouse models (YAC128, Hdh92 and Hdh111) at later stages (Guidetti et al., 2006).

These results suggest that an increased generation of QA may contribute, at least in part, to excitotoxicity in HD and that inhibition of this pathway may have beneficial effects.

Dysfunctional cortical glutamatergic neurons may cause a sustained glutamate stimulation of the vulnerable striatal neuronal populations. In agreement, striatal neurons from HD mice expressing one-third of the full-length huntingtin gene with 100 CAG repeats (HD100) displayed altered responses to cortical stimulation and to activation by NMDA, and multiple perturbations in corticostriatal synaptic function were recently observed in mice expressing the full-length human huntingtin gene with either 72 (YAC72) or 128 (YAC128) CAG repeats (Milnerwood and Raymond, 2007). Under conditions of chronic activation of NMDA receptors the intracellular Ca^{2+} concentration increases, which can have deleterious consequences including mitochondrial dysfunction, activation of the Ca^{2+}-dependent neuronal isoform of nitric oxide (NO) synthase (nNOS), generation of NO and other reactive oxygen species (ROS), activation of Ca^{2+}-dependent proteases such as calpains, and apoptosis (Rego and Oliveira, 2003). Importantly, mutant huntingtin can also sensitize the inositol (1,4,5)-triphosphate receptor type 1 (InsP3R1) located in the membrane of the endoplasmic reticulum, promoting a further increase in intracellular Ca^{2+} (Tang et al., 2003). Collectively, these events can contribute to the progressive neurodegeneration observed in the HD striatum. Accordingly, surgical decortication (lesion of the corticostriatal pathway) was recently shown to extend the survival and improve the behavioral, neuropathological and biochemical phenotype of R6/2 transgenic mice by significantly lowering striatal glutamate levels (Stack et al., 2007).

Neuroimaging analysis of human HD brains revealed cortical sensorimotor degeneration early in the disease, while microarray analysis found expression changes in the motor cortex but almost no changes in the frontal cortex or cerebellum, further

suggesting that a cortical dysfunction contributes to the human condition (Hodges et al., 2006).

The striatum also receives dopaminergic input from the substantia nigra pars compacta, also implicating this pathway in the selective striatal degeneration observed in HD. In support of this, several studies have shown degeneration of nigrostriatal projections, an atrophy of dopaminergic neurons in the substantia nigra and a striking reduction in the dopaminergic striatal neuronal population in HD brains (Gil and Rego, 2008). Aggregates of mutant huntingtin were also found in this brain region. Furthermore, a marked loss of tyrosine hydroxylase (the rate-limiting enzyme for dopamine biosynthesis) and a down-regulation of the DA transporter and the D_1 and D_2 dopamine receptors occurs in HD brains and R6/2 mice, probably due to altered gene transcription induced by mutant huntingtin (Gil and Rego, 2008). Interestingly, it was previously suggested that loss of D_2 dopamine receptors is a sensitive indicator of early neuronal impairment in preclinical carriers of the HD mutation (van Oostrom et al., 2005). Overall, these results suggest that both a presynaptic and a postsynaptic impairment may contribute to a dysfunctional nigrostriatal pathway.

The nigrostriatal dysfunction may be involved in causing the motor and cognitive deficits occurring in HD. Loss of both pre- and postsynaptic markers of dopamine neurotransmission is positively correlated with cognitive performance in asymptomatic and symptomatic HD patients (Bäckman and Farde, 2001). Furthermore, late stages of the disease are normally characterized by bradykinesia and rigidity, symptoms that may be directly caused by the progressive loss of dopamine receptors in the striatum.

In vitro and *in vivo* studies have also shown that dopamine itself can enhance polyglutamine toxicity. Using primary cultures of striatal neurons transiently expressing exon 1 of mutant huntingtin, it has been shown that low doses of dopamine act synergistically with mutant huntingtin to activate the proapoptotic transcription factor c-

Jun through a mechanism that is thought to involve the production of ROS and the activation of the c-Jun N-terminal kinase pathway. Importantly, dopamine also increased aggregate formation in this *in vitro* model, which could be reversed by a selective D_2 receptor antagonist (Charvin et al., 2005). In another recent study, double-mutant mice (generated by crossing the dopamine transporter knockout mouse with a knock-in HD mouse model expressing huntingtin with 92 CAG repeats) exhibited increased stereotypic activity at 6 months of age, followed by a progressive decline of their locomotor hyperactivity. In this hyperdopaminergic mouse model of HD the appearance of NIIs and neuropil aggregates was shown to occur much earlier and to a greater extent in the striatum and other dopaminergic brain regions as compared to control HD mice (Cyr et al., 2006). Thus, based on the results from these studies, it is reasonable to conclude that dopamine exacerbates mutant huntingtin aggregation both *in vitro* and *in vivo*, although the relationship between dopamine-mediated aggregation and striatal neurodegeneration needs to be clarified.

Importantly, dopamine itself can be a source of ROS and a trigger of oxidative stress. Under normal conditions, the enzyme monoamino oxidase converts dopamine into 3,4-dihydrophenylacetic acid (DOPAC) and hydrogen peroxide (H_2O_2). However, dopamine can also undergo a nonenzymatic process of auto-oxidation producing dopamine quinones and H_2O_2, and a dopamine semiquinone and superoxide (O_2^-). H_2O_2 and O_2^- can be further oxidized, respectively, through the Fenton (in the presence of transition metal ions) and the Haber–Weiss reactions, producing the extremely reactive hydroxyl radical.

It was recently shown that glutamate and DA signaling pathways may act synergistically to induce elevated Ca^{2+} signals and to cause the degeneration of medium spiny neurons in YAC128 (Tang et al., 2007).

Thus, the intracellular dysfunction of neurons (and glia) induced by mutant huntingtin results in the dysfunction of neuronal interactions and circuits. These dysfunctional cell–cell interactions will in turn exacerbate the dysfunction of individual neurons, thus forming a positive feedback loop that will ultimately culminate in cell death.

Studies in HD patients and HD post-mortem tissue have shown the following. (i) A significant decrease in glucose uptake (consumption) in the cortex (frontal and temporal lobes) and striatum (caudate and putamen) of both pre-symptomatic and symptomatic HD patients (Ciarmiello et al., 2006). (ii) A significant reduction in aconitase activity in the striatum (caudate and putamen) and cerebral cortex. Aconitase is an iron- and sulfur-containing enzyme of the tricarboxylic acid cycle and is thought to be particularly sensitive to inhibition by peroxinitrate ($ONOO^-$) and O_2^-. Thus, a reduction in its activity can be interpreted as an indirect indicator of ROS generation, mitochondrial dysfunction and excitotoxicity (Tabrizi et al., 1999). (iii) A significant decrease in the activities of mitochondrial complexes II–III and IV in the striatum. Contradictory results have also been published regarding the activity of mitochondrial complex I in HD platelets, with an initial study showing a striking reduction in the activity of this complex, and subsequent studies reporting no deficiencies in platelet mitochondrial function (Powers et al., 2007). Similarly, analysis of mitochondrial dysfunction in muscle from HD carriers revealed a defect of complex I, but in a more recent study no significant differences in the activities of complexes I and IV were found, although changes in the activity of complexes II–III were correlated with disease duration (Turner et al., 2007). (iv) An increase in lactate concentrations in the striatum and cerebral cortex (Koroshetz et al., 1997). (v) An increase in lactate/pyruvate ratio in the cerebrospinal fluid (Koroshetz et al., 1997). (vi) A reduced phosphocreatine/inorganic phosphate ratio in skeletal muscle and a significant delay in the recovery of phosphocreatine levels after exercise (a direct measure of ATP synthesis) in HD patients

and mutation carriers (Saft et al., 2005). (vii) Decreased mitochondrial ATP generation (Seong et al., 2005). (viii) Morphological and morphometric changes, as well as decreased membrane potential in mitochondria from lymphoblasts of both heterozygous and homozygous HD patients (Squitieri et al., 2006). (ix) Depletion of mitochondrial DNA in leukocytes from HD patients (Liu et al., 2008).

Interestingly, mutant huntingtin was also shown to disrupt mitochondrial-dependent Ca^{2+} handling, an effect that is ameliorated upon treatment with histone deacetylase inhibitors (Oliveira et al., 2006). On the other hand, mutant huntingtin can affect mitochondrial function by inhibiting the expression of PGC-1α (Weydt et al., 2006). Mutant huntingtin suppresses the activity of the promotor of the uncoupling protein-1 (UCP-1, the effector of adaptive thermogenesis) gene, an effect that can be reversed by PGC-1α (Weydt et al., 2006). Moreover, reduced expression of PGC-1α target genes in HD is related to impaired ATP production and a reduction in intact mitochondria, linking transcriptional deregulation and mitochondrial dysfunction in HD (Weydt et al., 2006). Importantly, crossbreeding of PGC-1α-knockout mice with HD knock-in mice exacerbates striatal neurodegeneration and motor abnormalities in the double-mutant mice, while lentiviral-mediated delivery of PGC-1α into the striatum of R6/2 mice has neuroprotective effects (Cui et al., 2006). Moreover, resveratrol, an activator of sirtuin Sir2 homolog 1 (SIRT1) was shown to modulate the SIRT1–PGC-1α pathway and to have a neuroprotective effect against mutant huntingtin-induced metabolic dysfunction (Parker et al., 2005). Indeed, SIRT1 functionally interacts with and deacetylates PGC-1α *in vitro* and *in vivo*, thus regulating energy and metabolic homeostasis. This may have some implications in the search for new HD therapeutic targets, as molecules that activate PGC-1α may be valuable in promoting mitochondrial function and striatal survival.

Taken together, these reports implicate mitochondrial dysfunction and metabolic impairment in HD pathology. Whether these energy deficits occur early on during the disease progression is still uncertain.

Systemic administration of the toxin 3-nitropropionic acid (3-NP; an irreversible inhibitor of mitochondrial complex II) has been largely shown to cause striatal neurodegeneration in rats and primates. Intrastriatal injection of this toxin or malonate (a reversible mitochondrial complex II inhibitor) in rats also leads to striatal neuronal loss, further implicating mitochondrial dysfunction in HD. Striatal neurodegeneration induced by 3-NP appears to be related to the early expression and activation of matrix metalloproteinase-9 by ROS which can digest the endothelial basal lamina, leading to the disruption of the blood–brain barrier and to progressive striatal damage (Kim et al., 2003). Excitotoxicity and dopamine toxicity were previously shown to modulate the striatal degeneration induced by 3-NP, supporting the idea that this mitochondrial toxin and mutant huntingtin may have certain degeneration mechanisms in common.

Mitochondria are also central players in the process of caspase activation and apoptosis. 3-NP causes mitochondrial-dependent apoptotic neuronal death through the release of cytochrome c and consequent activation of caspases, or the release of apoptosis-inducing factor (Almeida et al., 2006). Experiments performed with HD lymphoblasts demonstrated that these HD cells show a robust mitochondrial membrane depolarization when exposed to weak stress conditions whereas myoblasts from presymptomatic and symptomatic HD subjects show mitochondrial depolarization, cytochrome c release, increased caspase-3, -8 and -9 activities, and defective cell differentiation (Ciammola et al., 2006). Furthermore, it was demonstrated that huntingtin associates with the outer mitochondrial membrane and that a truncated mutant huntingtin fragment can directly induce the opening of the mitochondrial permeability transition pore (MPTP) in isolated mouse liver mitochondria, an effect that was accompanied by a significant release of

cytochrome c (Choo et al., 2004). Cytochrome c leads to caspase activation which in turn can cleave mutant huntingtin and promote its translocation into the nucleus, where it abnormally interacts with several transcription factors. Importantly, p53, a transcription factor that regulates the expression of various mitochondrial proteins such as Bax, interacts with mutant huntingtin both in HD lymphoblasts and in neuronal cells expressing mutant huntingtin. In neuronal cultures, mutant huntingtin induces an upregulation of the nuclear levels of p53 and an increase in its activity. Interestingly, p53 levels are also increased both in HD transgenic mice and in HD patients, and genetic deletion of p53 suppresses neurodegeneration in HD transgenic flies and neurobehavioral abnormalities of HD transgenic mice (Bae et al., 2005). Thus, it is likely that mutant huntingtin-induced increase in p53 activity induces further mitochondrial abnormalities that contribute to HD.

Importantly, mitochondrial dysfunction is also the major contributor to oxidative stress which, along with the excitotoxic activation of nNOS and the metabolism of dopamine, can lead to a toxic increase in the levels of ROS. Susceptible neurons, as in the case of HD, may not be able to handle well an increase in ROS production. High ROS levels may promote intracellular cascades of oxidative stress by oxidizing proteins and DNA and triggering lipid peroxidation. Therefore, it is reasonable to speculate that oxidative stress might play a crucial role in the neurodegenerative process of HD. In support of this hypothesis, several studies have shown an altered pattern of expression and activity of the enzyme nitric oxide synthase (NOS), as well as of the antioxidants superoxide dismutase (SOD) and ascorbate in R6/1 and R6/2 transgenic mice respectively. Furthermore, ROS production is increased in the striatum of R6/1 mice. Moreover, it has been suggested that NO generation is the underlying cause of the observed inhibition of aconitase in human HD brains. These authors suggest that aconitase inhibition is then followed by the inhibition of complex II–III and the initiation of a

self-amplifying cycle of ROS generation that results in severe ATP depletion and cell death (Tabrizi et al., 1999). Evidence of oxidative damage has been detected in peripheral tissues of human HD patients, including increased leukocyte levels of 8-hydroxydeoxyguanosine (8-OHdG), increased plasma levels of malondialdehyde, decreased activities of the antioxidant enzymes Cu–Zn-superoxide dismutase (SOD1) and glutathione peroxidase (GPx) in erythrocytes, and a decreased catalase activity in skin fibroblasts (Chen et al., 2007). In addition, mitochondrial-dependent generation of ROS in HD seems to be due, at least in part, to suppression of PGC-1α in the presence of mutant huntingtin, as this transcription coactivator is required for the induction of ROS-detoxifying enzymes, namely Mn-superoxide dismutase (SOD2) and GPx, implicating PGC-1α as an important protector against oxidative damage in HD.

There is strong evidence suggesting that aging is associated with poor handling of ROS by neuronal cells (Lu et al., 2004). Thus aging might, at least in part, increase mutant huntingtin toxicity through increased oxidative stress.

Mutant huntingtin is a substrate for several caspases and calpains, which are believed to mediate the formation of the toxic N-terminal fragments. Moreover, sequestration of pro-caspases in the aggregates is thought to promote their activation, thus promoting an intracellular cascade of proteolytic events. Furthermore, calpain, caspase-1 and caspase-8 activity is increased in HD brains (Gil and Rego, 2008). These findings have led to the hypothesis that an apoptotic mechanism is responsible for HD neuronal loss. Indeed, early studies identified apoptotic-like cells in the HD striatum with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). However, some of these studies failed to detect the characteristic pattern of DNA laddering after DNA electrophoresis that is typically observed in cells undergoing apoptosis (Portera-Cailliau *et al.*, 1995). Thus, although the authors concluded that the presence of TUNEL-positive cells in the HD striatum strongly suggests the occurrence of apoptosis,

the lack of the characteristic pattern of apoptotic DNA fragmentation argues against it. Furthermore, the detection of activated astrocytes in HD brains suggests neuroinflammation, which is generally absent in apoptotic conditions. Therefore, the evidence for a pure apoptotic process contributing to HD cell death is controversial. Nevertheless, the activation of certain apoptotic pathways is likely to contribute to some degree to HD pathology (Hickey and Chesselet, 2003).

Some evidence suggests that autophagy may mediate cell loss in HD. HD brains display endosomal and/or lysosomal organelles, multivesicular bodies and lipofuscin accumulation reminiscent of autophagy (Sapp et al., 1999). Moreover, several studies have shown that expression of mutant huntingtin induces endosomal and/or lysosomal activity and sequestration of the mammalian target of rapamycin (mTOR; a protein kinase involved in the regulation of autophagy) into the aggregates of mutant huntingtin with subsequent inhibition of its kinase activity. These events lead to autophagy, which in turn may promote huntingtin degradation and the clearance of aggregates. Within this scenario, it was recently shown that rapamycin and several small-molecule enhancers of rapamycin, which seem to act either independently or downstream of the target of rapamycin, are able to enhance the clearance of mutant huntingtin fragments and attenuate their toxicity in various HD models. Moreover, clearance of accumulated mutant exon 1 huntingtin is also triggered by the activation of the insulin signaling pathway, despite the activation of Akt, mTOR and S6 kinase, in a process requiring Beclin1 and hVps34, two proteins implicated in macroautophagy, the latter a class III phosphatidylinositol 3-kinase (Yamamoto et al., 2006).

Hence, autophagy may represent an initial attempt of the HD cell to eliminate the mutant protein that over the course of the disease becomes overloaded, insufficient and dysfunctional, eventually resulting in cell degradation. Indeed, the lysosomal proteases cathepsins D, B and L were recently shown to promote the generation and accumulation

of mutant huntingtin fragments (Kim et al., 2006). Furthermore, sequestration of Beclin 1 into NIIs might reduce the autophagic clearance of mutant huntingtin. Finally, some evidence suggests that molecular determinants of autophagic vacuole formation are more easily recruited to cytoplasmic than to nuclear aggregates, which could help to explain the toxicity of nuclear inclusions in HD (Iwata et al., 2005).

TREATMENT OF HUNTINGTON'S DISEASE

Despite the extraordinary growth of knowledge about the cellular mechanisms involved in HD-related neurodegeneration, no effective neuroprotective or disease-modifying therapy has emerged. The selection of the most appropriate symptomatic treatment depends on the most dominant or troublesome symptom. Patients with HD may exhibit a variety of movement disorders, with the most common being chorea, but also parkinsonism (characteristic of juvenile HD), ataxia, dystonia, bruxism, myoclonus, tics, and tourettism. The psychiatric, behavioral, and cognitive manifestations of the disease are also very debilitating, rendering the patient dependent on others. These include irritability, depression, lack of motivation, paranoia, hallucinations, and cognitive and intellectual decline. Other contributing symptoms to the overall disability are dysphagia, weight loss, dysarthria, and sleep disturbance. Several clinical rating scales have been developed and validated, and have been used extensively in clinical trials to ensure a uniform assessment of outcomes. The Unified Huntington's Disease Rating Scale (UHDRS), developed by the Huntington Study Group (HSG), comprises several subscales that assess motor function, cognition, behavior, functional status, and independence (Huntington study group, 1996). The Abnormal Involuntary Movement Scale (AIMS) uses a rating of 0 (no movement) to 4 (severe) to quantify abnormal movements. Scores are attributed separately for abnormal movements affecting the muscles of facial expression, lips and perioral area, jaw, tongue, upper extremities,

lower extremities, and trunk. The scale also incorporates other items, related to the patient's awareness of and disability caused by the abnormal movements, and the presence of dentures or edentia. The functional status of HD patients is commonly assessed using the Total Functional Capacity Scale (TFC) and the Independence Scale (IS), both of which are incorporated into the UHDRS.

Because neuroprotective treatments remain elusive, improving the quality of life of HD patients is a priority. Traditionally, neuroleptics and dopamine depleters have been the cornerstone of symptomatic therapy in HD (Adam and Jankovic, 2008). The atypical neuroleptics tend to be used more frequently in the treatment of HD. Their advantage over the typical neuroleptics is a diminished incidence of the adverse effects familiar in movement disorders (acute dystonia, akathisia, parkinsonism, tardive dyskinesia). The atypical neuroleptics are not necessarily more effective, however, and they may cause more weight gain and other metabolic side effects (e.g., dyslipidemia, increased insulin resistance), resulting in a similar treatment discontinuation rate10 as with typical neuroleptics. Typical neuroleptics, including haloperidol, pimozide, fluphenazine, thioridazine, sulpiride, and tiapride, have been used for the treatment of psychotic symptoms as well as chorea in HD (Adam and Jankovic, 2008). These drugs have a high affinity for D_2 dopamine receptors, however, and can potentially induce parkinsonism, akathisia, acute dystonias, and tardive dyskinesia. Several other classes of medications have been used to ameliorate the various symptoms of HD, including antidepressants, antiglutamatergic drugs, GABA agonists, antiepileptic medications, acetylcholinesterase inhibitors, and botulinum toxin. Recently, surgical approaches including pallidotomy, deep brain stimulation, and fetal cell transplants have been used for the symptomatic treatment of HD. The selected therapy must be customized to the needs of each patient, minimizing the potential adverse effects (Adam and Jankovic, 2008).

FRIEDREICH'S ATAXIA

Friedreich's ataxia (FRDA) was first reported in 1863 by Nicholaus Friedreich in Heidelberg, Germany. He described the essential findings of the disease: degenerative atrophy of the posterior columns of the spinal cord leading to progressive ataxia, sensory loss, and muscle weakness, often associated with scoliosis, foot deformity, and heart disease. Although it was initially reported more than 150 years ago, the complete clinical spectrum of FRDA and the features that distinguished this disease from other ataxia syndromes have been controversial. The Québec Collaborative Group in 1976 (Geoffreoy et al., 1976) and Harding in 1981 (Harding et al., 1981) defined the essential clinical criteria for diagnosis of FRDA.

The FRDA gene was localized to chromosome 9q, and the most common mutation was defined as an unstable expansion of a GAA trinucleotide repeat sequence. Since this discovery, the molecular diagnosis of atypical cases has become possible and the phenotypic spectrum of FRDA has been broadened (Pandolfo M, 2002). FRDA is the most common hereditary ataxia. It is an autosomal-recessive neurodegenerative disorder with an estimated prevalence of 1:50000 (Delatycki et al., 2000).

Progressive, unremitting ataxia is the principal feature of this disease. Most commonly, it begins with clumsiness in gait. The onset is usually around puberty, but it may vary from 2-3 years of age to later than 25 years of age (Pandolfo M, 2002). The essential clinical features are autosomal-recessive inheritance, onset before 25 years of age, progressive limb and gait ataxia, absent tendon reflexes in the lower extremities, electrophysiologic evidence of axonal sensory neuropathy followed by (within 5 years of onset), dysarthria, areflexia at all four limbs, distal loss of position and vibration sense, extensor plantar response, and pyramidal weakness of the legs (Alper and Narayanan, 2003). Loss of ambulation occurs on average 15.5 ± 7.4 years after disease onset (Delatycki et al., 2000). Cardiomyopathy is the most common cause of death.

Patients who exhibit all these clinical features listed by Harding are considered to have the "typical" or "classic" form of the disease.

As with age of onset, there is a great variability in other clinical features, including the rate of the progression, severity, and extent of the disease involvement. Cardiac complications may be severe enough to cause early death or may be minimal or absent. Other variable neurologic findings include oculomotor abnormalities, nystagmus, optic atrophy, and sensorineural hearing loss (Pandolfo M, 2002). The most common abnormality of ocular movement is fixation instability. Ophthalmoparesis is not observed. More than half the patients manifest progressive scoliosis, and about half manifest pes cavus, pes equinovarus, and clawing of the toes (Harding et al., 1981). Amyotrophy of the small muscles of the hand and distal muscles of the leg and foot is common. About 10% of FRDA patients are diabetic, and most diabetic patients require insulin therapy. Cardiomyopathy is evident in around two thirds of patients with FRDA and is primarily symmetric concentric hypertrophic cardiomyopathy, although some patients exhibit asymmetric septal hypertrophy (Alper and Narayanan, 2003).

AIM OF THE THESIS

Huntington's disease (HD) is a dominantly inherited neurodegenerative disorder characterized by progressively worsening chorea, psychiatric disturbances and cognitive impairment. The degenerative process primarily involves medium spiny striatal neurons and, to a lesser extent, cortical neurons. Of importance, GABAergic enkephalin neurons of the basal ganglia are the most vulnerable in HD and their early dysfunction is responsible for the development of chorea. HD is caused by the expansion of a polymorphic CAG trinucleotide repeat encoding a poly-glutamine tract within a protein named huntingtin. The mechanisms responsible for mutant huntingtin pathogenicity are still largely unknown. Both gain of function (in which mutant huntingtin has toxic properties) and loss of function of normal huntingtin have been proposed. Furthermore, it is unknown why the striatal medium spiny neurons are most vulnerable in HD, despite ubiquitous expression of mutant and normal huntingtin.

It is noteworthy that adenosine A_{2A} and dopamine D_2 receptors are co-expressed on striatal GABAergic enkephalin neurons. In these cells, A_{2A} adenosine receptors (A_{2A}ARs) stimulate while dopamine D_2 receptors inhibit adenylyl cyclase and cAMP formation. Adenosine is thought to act as a trigger for development-associated apoptosis and, under specific conditions, activation of the A_{2A}ARs may result in cell death. Conversely, under certain circumstances, A_{2A}ARs may mediate trophic effects. Dysregulation of A_{2A}AR physiology may thus account for striatal cell loss in HD.

On this background, the aim of this study is to investigate the role of A_{2A}ARs in Huntington's disease. In particular we have evaluated the expression and functionality of the A_{2A}ARs in different in vitro and in vivo models of Huntington's disease as well as of other related polyglutamine disease.

The aim of the **chapter 1** was to investigate if the presence of mutant huntingtin may alter $A_{2A}AR$ signaling in striatal cells ST14A. For this aim, the presence and the functional responses of $A_{2A}AR$ were evaluated in parental ST14A cells and stable subclones expressing either 548 aminoacids N terminal to the protein huntingtin in the wild-type (N548wt) or mutant (N548mu) versions, or the full-length wild-type (FLwt) and full-length mutant (FLmu). The expression of $A_{2A}AR$ was investigated through reverse transcriptase-polymerase chain reaction (RT-PCR) and receptor binding assays.

The $A_{2A}AR$ is coupled to a stimulatory protein Gs that activate adenylil cyclase with the conseguent production of cAMP. In order to evaluate the functionality of $A_{2A}AR$ in parental ST14A and in subclones expressing mutant huntingtin, cAMP production assays were performed. The basal and forskolin stimulated level of cAMP were evaluated in all the striatal cell clones. Furthermore, it was studied the potency of typical $A_{2A}AR$ agonists to stimulate the production of cAMP as well as the effect of $A_{2A}AR$ antagonist to counteract this stimulation in both parental and huntingtin-expressing cells.

The aim of the **chapter 2** was to assess the presence of the aberrant $A_{2A}ARs$ phenotype in the basal ganglia of transgenic R6/2 mice, an animal model of Huntington's disease that express exon 1 of the human huntingtin gene with an expanded CAG repeat length of 141-157 under the influence of the human promoter. To study the expression of $A_{2A}ARs$, experiment of quantitative real time PCR and receptor binding assays were performed in striatal membranes of R6/2 mice at various presymptomatic and symptomatic stages. To assess the receptor activity, we performed ex-vivo measurements of cAMP production in homogenates from striata in the absence (basal activity) or presence of the selective $A_{2A}AR$ agonist CGS 21680, or after direct activation of the enzyme with forskolin.

The study described in **chapter 3** was undertaken to assess the status of the $A_{2A}AR$ in peripheral blood cells of HD-affected and non-affected mutation carrier subjects compared with control healthy individuals.

Two strategies were adopted to assess the status of the $A_{2A}AR$ in peripheral cells from HD subjects. The first one aimed at measuring the binding kinetic parameters of the $A_{2A}AR$ ligand [^{3}H]-ZM 241385 in platelets, lymphocytes and neutrophils of patients with Huntington's disease and non-affected mutation carrier subjects compared with control subjects. The second one aimed at assessing the ability of $A_{2A}AR$ agonists to stimulate adenylyl cyclase activity and cAMP formation in the blood cells of the three group of subjects.

The aim of the **chapter 4** was to extend the analysis of $A_{2A}AR$ activity in patients with Huntington's disease including a more detailed transversal study where different disease stages were represented, as well as in a larger number of pre-symptomatic subjects enrolled from different clinical sites. In addition, we tested whether the $A_{2A}AR$ dysfunction was present in other inherited neurological diseases, such as other polyglutamine- (Spinocerebellar Ataxia, SCA, type 1 and 2) or not polyglutamine-linked (Freidreich Ataxia, FRDA) neurodegenerative disorders. The identification in peripheral cells of a measurable significant change in a biological process might arguably serve as a useful endpoint to measure the efficacy of therapeutics targeting pathological mechanisms initiated by the mutation.

CHAPTER 1:

Aberrant amplification of A_{2A}AR signaling in striatal cells expressing mutant huntingtin.

INTRODUCTION

Huntington's disease (HD) is a dominantly inherited neurodegenerative disorder featuring progressive worsening chorea, psychiatric disturbances, and cognitive impairment due to brain cell loss, with basal ganglia showing the most dramatic morphological abnormalities and degeneration (Ross, 1995). The disease is caused by expansion of a polymorphic CAG trinucleotide repeat encoding of a poly-glutamine tract in Huntingtin (Htt), a 3154 aminoacids protein with recently described anti-apoptotic functions. The mechanisms responsible for mutant Htt pathogenicity are still largely unknown, but may involve a proteolytic cleavage of the protein to generate fragments that aggregate into the nucleus and cytoplasm (Wellington et al., 2000). Much less clear is the mechanism by which, despite ubiquitous expression of mutant (and normal) Htt in HD patients, selective degeneration of striatal medium spiny neurons occurs in the disease. Adenosine agonists induce potent psychomotor depression and inhibit the locomotor activity induced by dopamine agonists (Ferrè et al., 1993). This adenosine-dopamine interaction mainly depends on a postsynaptic $A_{2A}AR$-D_2 receptor interaction in striatum (Ferrè et al., 1993). In this brain area, $A_{2A}ARs$ are coexpressed with D_2 receptors on GABAergic enkephalin neurons, which are key components of the "indirect" striatal efferent pathway involved in regulation of motor activity, and are highly affected in HD (Reiner et al., 1988). By activating $A_{2A}ARs$ on these neurons, adenosine acts as a dopamine functional antagonist. It is interesting that $A_{2A}ARs$ stimulate and D_2 receptors inhibit adenylyl cyclase, which represents a key target in mediating the opposite effects of these two transmitters (Ferrè et al., 1993). Therefore, the possibility remains strong that changes in the adenosine-dopamine system in striatum play a role in the motor dysfunction typical of HD. It has been suggested that adenosine triggers development-associated apoptosis; under specific conditions, activation of the $A_{2A}ARs$ may result in cell death (Jacobson et al.,

1999). It may hence be hypothesized that a dysregulation of adenosine-mediated cell death, likely due to an alteration of $A_{2A}AR$ expression or function, may contribute to neurodegeneration in HD.

In this part of the study it has been tested the possibility that mutant Htt may alter $A_{2A}AR$ signaling in striatal cells expressing mutant Htt. In particular the presence and the functionality of $A_{2A}ARs$ have been studied through reverse transcriptase-polymerase chain reaction, receptor binding assays and cyclic adenosine monophosphate (cAMP) assays.

MATERIALS AND METHODS

Materials

SCH 58261 and [^{3}H]-SCH 58261 (specific activity 68.6 Ci/mmol) were from the Schering-Plough Research Institute, Milan, Italy. [^{3}H]-ZM 241385 (specific activity 17 Ci/mmol) was from Tocris Cookson Ltd. (Bristol, England). [^{3}H]-cAMP (specific activity 21 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, Bukinghamshire, England). NECA (5'-Nethylcarboxamidoadenosine), Ro 20-1724, (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone), GTPγS, cAMP, and forskolin were from Sigma Chemicals Co. (St. Louis, Mo.). Aquassure and Atomlight were from NEN Research Products (Boston, Mass.). All other reagents were of analytical grade and obtained from commercial sources.

Cell Culture

Parental ST14A cells and stable subclones expressing either 548 aminoacids N terminal to the protein huntingtin in the wild-type (N548wt) or mutant (N548mu) versions, or the full-length wild-type (FLwt) and full-length mutant (FLmu) proteins were grown at

33°C in the presence of Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum as previously described (Cattaneo and Conti, 1998). All transfected subclones have been previously demonstrated to express the exogenous proteins at comparable levels at least up to 25 passages in culture (Rigamonti et al., 2000). Expression levels are routinely checked by Western blot in early passaged cells. Cells were used when 80% confluence was reached.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from cells and tissue using Triazol Reagent (Gibco, BRL, Rockville, Md.). Retrotranscription of RNA was performed with SuperScript II (Gibco, BRL, Rockville, Md.) according to the instructions of the manufacturer and using 250 ng of random primers. RNAseOUT (20 U) (Gibco, BRL) was added to each reaction. cDNA (0.5 µg) was amplified by PCR using DyNAzyme EXT (Finnzymes) and primers specific for the $A_{2A}AR$ ($A_{2A}AR$ Fw: 5'-TGTCCTGGTCCTCACGCAGAG-3'; $A_{2A}AR$ Rev: 5'CGGATCCTGTAGGCGTAGATGAAGG-3'). PCR was carried out for 40 cycles with the following parameters: denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 7 min.

Receptor binding studies

Cells were washed with phosphate buffered saline and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with Polytron, and the homogenate was spun for 10 min at 1,000 g. The supernatant, collected in pools from 6 to 8 culture dishes, was then centrifuged for 30 min at 48,000 g, and each pool was used to perform a single saturation experiment. The membrane pellet was resuspended in 50 mM Tris HCl, 120 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4, incubated with 2 I.U./ml of adenosine deaminase

for 30 min at 37°C and centrifuged for 30 min at 48,000 g. The resulting pellet was resuspended at a concentration of 100–150 µg protein/100 µl, and this homogenate was used for assaying binding of either [^{3}H]-SCH 58261 (5-amino-7-(phenylethyl)-2- (2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) (Varani et al., 1997) or [^{3}H]-ZM 241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4] triazolo[2,3a][1,3,5] triazinyl-amino] ethyl)phenol). The protein concentration was determined according to a Bio Rad method (Bradford, 1976) with bovine serum albumin as a standard reference. Binding assays were carried out as previously described (Varani et al., 1997). In saturation studies, cell membranes were incubated for 60 min at 4°C with 8–10 different concentrations of either [^{3}H]-SCH 58261 or [^{3}H]-ZM 241385 (0.05–10 nM) in a total volume of 250 µl containing 50 mM Tris HCl, 120 mM NaCl, 5 mM KCl, 10 mM MgCl$_2$, and 2 mM CaCl$_2$, pH 7.4. Nonspecific binding was defined as binding in the presence of 10 µM NECA and was about 40% of total binding. Bound and free radioactivity was separated by filtering the assay mixture through Whatman GF/B glass-fiber filters by using a Micro-Mate 196 cell harvester (Packard). The filterbound radioactivity was counted by using a microplate scintillation counter (Top Count) at an efficiency of 57% with Micro Scint 20. A weighted nonlinear least-squares curve-fitting program LIGAND (Munson and Rodbard, 1980) was used for computer analysis of saturation experiments.

Measurement of cAMP levels

Cells were washed in phosphate buffered saline and centrifuged for 10 min at 200 g. The supernatant was discarded, the pellet was resuspended in a buffer containing 120 mM NaCl, 5 mM KCl, 0.37 mM NaH$_2$PO$_4$, 10 mM MgCl$_2$, 2 mM CaCl$_2$, 5 g/L D-glucose, 10 mM Hepes-NaOH, pH 7.4, and centrifuged again for 10 min at 200 g. Cells

$(4 \times 10^5$ cells/tube) were then suspended in 0.5 ml incubation mixture (120 mM NaCl, 5 mM KCl, 0.37 mM NaH$_2$PO4, 10 mM MgCl$_2$, 2 mM CaCl$_2$, 5 g/L D-glucose, 10 mM Hepes-NaOH, pH 7.4) containing 1.0 I.U. of adenosine deaminase/ml and 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 201724, as phosphodiesterase inhibitor), and preincubated for 10 min in a shaking bath at 37°C. Then forskolin (1 μM) or NECA (1 nM-10 μM) was added to the mixture, and the incubation continued for a further 5 min. The effect of several A$_{2A}$AR antagonists such as CGS 15943 (1 μM), ZM 241385 (1 μM), and SCH 58261 (1 μM) was determined by antagonism of NECA (100 nM)-induced stimulation of cyclic AMP levels. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The TCA suspension was centrifuged at 2,000 g for 10 min at 4°C, and the supernatant was extracted four times with water-saturated diethyl ether. The final aqueous solution was tested for cyclic AMP levels by a competition protein- binding assay carried out as previously described (Varani et al., 1998). Samples of cyclic AMP standards (0–10 pmol) were added to each test tube containing trizma base 0.1 M, aminophylline 8.0 mM, 2 mercaptoethanol 6.0 mM, pH 7.4, and [^{3}H]-cAMP in a total volume of 0.5 ml. The binding protein, previously prepared from beef adrenals, was added to the samples previously incubated at 4° C for 150 min. The samples, after the addition of charcoal, were centrifuged at 2,000 g for 10 min. The clear supernatant (0.2 ml) was mixed with 4 ml of Ultimagold XR (Hewlett-Packard) and counted in a LS-1800 Beckman scintillation counter.

RESULTS

Presence of the A$_{2A}$ARs in ST14A cells and in Htt expressing subclones.

Striatal ST14A cells have been previously stably transfected with either normal or mutant full-length and truncated Htt constructs to generate a large number of stable

subclones (Rigamonti et al., 2000). The rationale for extending the analysis to the phenotype of cells expressing truncated Htts derives from well established data demonstrating that N-terminal fragments resulting from proteolytic cleavage of full-length mutant Htt are more toxic than the full-length protein itself (Wellington et al., 2000). Figure 1.1 shows $A_{2A}ARs$ mRNA levels in parental ST14A cells and in the various stable subclones expressing N548wt, N548mu, FLwt, and FLmu cDNAs. As a positive control, mRNAs from adult rat striatum, cortex, and $A_{2A}AR$ stably transfected CHO cells were loaded. As expected, RTPCR revealed an enrichment of the $A_{2A}ARs$ in striatum and in transfected CHO cells, with lower levels in rat cortex, in agreement with previous reports on the brain distribution of this receptor subtype (Fredholm et al., 2001). Both parental ST14A cells, which are striatum-derived, and all stable subclones express the 630 bps band corresponding to the $A_{2A}AR$ mRNA, although to a different extent. Despite differences in mRNA levels, comparable $A_{2A}AR$ protein densities are found in all clones, as shown by radio-ligand binding studies performed with the highly selective $A_{2A}AR$ antagonist [^{3}H]-SCH 58261 (Table 1.1). Scatchard plot analysis revealed the presence of a single class of specific, high-affinity, and saturable binding sites in both ST14A and in Htt-expressing cells, with comparable Bmax values, independently of the presence of normal or mutant Htt (Table 1.1). K_D values were instead significantly reduced in FLmu cells with respect to parental ST14A cells, a change that was even more significant in N548mu cells (Table 1.1). Comparable data were obtained with the $A_{2A}AR$ ligand [^{3}H]-ZM 241385. For the ST14A, FLwt, N548wt, Flmu, and N548mu, K_D values were, respectively, 2.30 ± 0.10; 2.42 ± 0.08; 2.48 ± 0.05; 1.61 ± 0.06*; 1.32 ± 0.06** nM (* $P<0.05$ vs. control; ** $P<0.01$ vs. control); corresponding Bmax values were 92 ± 6; 90 ± 8; 90 ± 6; 93 ± 5; 90 ± 3 fmol/mg protein. No changes of K_D values were detected in clones expressing either FLwt or N548wt (Table 1.1). These data suggest a selective increase of binding affinity in the

presence of mutant Htt. Figure 1.2 shows an example of saturation analysis performed with [^{3}H]-SCH 58261 (0.5–10 nM) in parental ST14A cells and in N548mu (A) or FLmu (B) cells. Consistent with the K_D values reported in Table 1.1, at low radioligand concentrations (0.5–4.5 nM), an increase of binding values was detected in both N548mu and FLmu cells, as demonstrated by the shift to the left of [^{3}H]-SCH 58261 saturation curves (Fig. 1.2A, B).

Selective alteration of forskolin-stimulated adenylyl cyclase response in cells expressing mutant Htt.

The A_{2A}ARs is coupled to stimulation of adenylyl cyclase via Gs stimulatory proteins, which leads to increases of cAMP formation (Fredholm et al., 2001). We therefore analyzed the sensitivity of adenylyl cyclase to pharmacological agents that are known to specifically activate this effector system. Despite no changes of basal enzyme activity (Table 1.2), response of adenylyl cyclase to the direct activator forskolin (used in the presence of the cAMP-dependent phosphodiesterase inhibitor RO 201724) was notably increased in N548mu (and Flmu) cells with respect to parental or wild-type Htt cells. As shown in Table 1.2, an amplification of cAMP formation was already demonstrable with forskolin alone in N548mu cells. To shed light on the cyclase alteration observed in cells expressing mutant Htt, we performed a Michaelis-Menten analysis of adenylyl cyclase activity in ST14A and N548mu cells. We therefore incubated cell homogenates in the presence of a fixed forskolin concentration and in the presence of graded concentrations of substrate (0.001–1000 μM ATP, see x-axis of Fig. 1.3A). Analysis of the data reported in Figure 3A, according to Lineweaver-Burk (Fig. 1.3B), shows a statistically significant reduction of enzyme Km value in N548mu cells with respect to parental cells (82±3 nM vs. 180±4 nM, respectively). No changes of Vmax values were detected (17.2±0.6 pmol/min in N548mu compared with 16.8±0.4 pmol/min in ST14A

cells). This alteration in cyclase activity appears to specifically involve the catalytic subunit of the enzyme, because no changes in cAMP production were observed in the presence of GTPγS, a direct G protein activator. For example, cAMP formation in N548mu incubated with 10 μM GTPγS was 65 ± 2 pmoles/10^5 cells compared with 64 ± 1 pmoles/10^5 cells in ST14A cells.

Aberrant amplification of $A_{2A}AR$-dependent adenylyl cyclase response in cells expressing mutant Htt.

When the adenosine analog NECA was applied to the cells, an aberrant amplification of adenylyl cyclase response was detected selectively in cells expressing mutant Htt. In all clones, NECA (0.1 nM–10 μM) significantly increased cAMP production in a concentration-dependent manner. However, as shown in Figure 1.4, which reports the log dose-response curve for NECA in cells expressing either N548wt and FLwt (A) or N548mu and FLmu (B), cAMP formation was significantly higher in the latter two at almost all agonist concentrations with respect to parental ST14A cells (EC_{50} values: 198 ± 15*; 93 ± 9** nM in FLmu and N548mu, respectively, versus 270 ± 10 in ST14A cells; * $P<0.05$; **$P<0.01$ vs. control). No differences of NECA responses were detected in FLwt and N548wt cells with respect to ST14A cells at any of the utilized agonist concentrations (EC_{50} values: 253 ± 11 and 236 ± 13, respectively). These results suggest a selective increase of responsiveness of the $A_{2A}AR$/adenylyl cyclase system in the presence of mutant Htt. This change is unrelated to modifications of endogenously produced adenosine because adenosine deaminase was present during the adenylyl cyclase assay. Selective $A_{2A}AR$ antagonists counteracted $A_{2A}AR$-stimulated cAMP formation in both parental and Htt-expressing cells. To further confirm that the effect induced by NECA on cAMP formation is due to stimulation of the $A_{2A}AR$ subtype, we performed experiments in the presence of adenosine receptor antagonists characterized

by various degree of selectivity towards the A_{2A}ARs subtype. As shown in Table 1.3, in ST14A cells, both the relatively selective antagonists CGS15943 and ZM 241385 and the highly selective antagonist SCH 58261, completely prevented the increases of cAMP induced by NECA at an agonist concentration (100 nM), which selectively stimulates adenylyl cyclase via the A_{2A}AR. Remarkably, all the tested antagonists retained the ability to counteract NECA-induced cAMP formation also in cells expressing mutant Htt, where adenylyl cyclase activity is aberrantly increased with respect to ST14A and to wild-type Htt cells.

Table 1.1 - Binding parameters of the $A_{2A}AR$ ligand [^{3}H]-SCH 58261 in membranes of ST14A, wild-type and mutant Htt cells.

	ST14A	FL wt	N548 wt	FL mu	N548 mu
K_D (nM)	2.20 ± 0.06	2.36 ± 0.11	2.38 ± 0.27	$1.96 \pm 0.06*$	$1.34 \pm 0.08**$
Bmax (fmol/mg protein)	92 ± 3	89 ± 6	89 ± 4	94 ± 5	92 ± 2

Analysis by Student's t test; * P <0.05 vs control; ** P <0.01 vs control.

Table 1.2 - Basal and forskolin-stimulated cAMP formation in ST14A, wild-type and mutant Htt cells.

	ST14A	FL wt	N548 wt	FL mu	N548 mu
Basal levels	18 ± 2	19 ± 2	18 ± 1	20 ± 2	20 ± 1
Forskolin (1 µM)	45 ± 5	54 ± 6	47 ± 3	55 ± 5	$72 \pm 4*$
Forskolin (1 µM) + RO 201724 (0.5 mM)	58 ± 4	60 ± 6	63 ± 6	78 ± 7	$88 \pm 5*$

Formation of cAMP was detected in the absence of stimuli (basal levels), and upon stimulation with forskolin, in the absence or presence of the cAMP -phosphodiesterase inhibitor Ro 201 724. Data are expressed in pmol/10^5 cells.
Analysis was by Student's t test; $* P < 0.01$ vs control.

Table 1.3 - Effect of different $A_{2A}AR$ antagonists on NECA-stimulated cAMP formation in ST14A, wild-type and mutant Htt cells.

	ST14A	FL wt	N548 wt	FL mu	N548 mu
Basal cAMP levels	18 ± 2	19 ± 2	18 ± 1	20 ± 2	20 ± 1
NECA-stimulated cAMP formation	38 ± 5	39 ± 5	40 ± 4	48 ± 3	55 ± 6
NECA + CGS 15943 (1 μM)	$19 \pm 3*$	-	-	-	$22 \pm 4*$
NECA + ZM 241385 (1 μM)	$20 \pm 4*$	-	-	-	$21 \pm 3*$
NECA + SCH 58261 (1 μM)	$18 \pm 3*$	$19 \pm 3*$	$18 \pm 2*$	$19 \pm 1*$	$19 \pm 2*$

Formation of cAMP was detected in the absence of stimuli (basal levels) and upon stimulation with 100 nM NECA, in the absence or presence of the indicated A_{2A} receptor antagonists. Data are expressed as $pmol/10^5$ cells. Analysis was by Student's t test; * P <0.01 vs NECA-stimulated cAMP formation.

Figure 1.1 - RT-PCR for the $A_{2A}AR$ in parental ST14A cells and subclones expressing N548wt, N548mu, FLwt, and FLmu cDNAs.
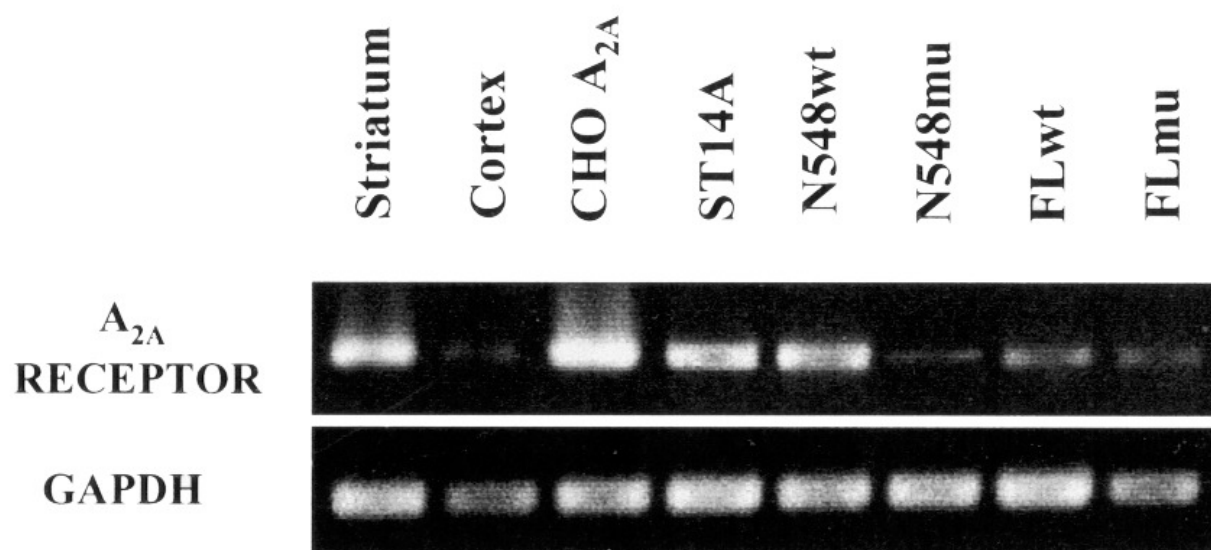
Figure 1.2 - Saturation curves and Scatchard plot of [³H]-SCH 58261 specific binding to ST14A (•), N548 mu (▪) (A) and ST14A (•), FL mu (▲) (B).
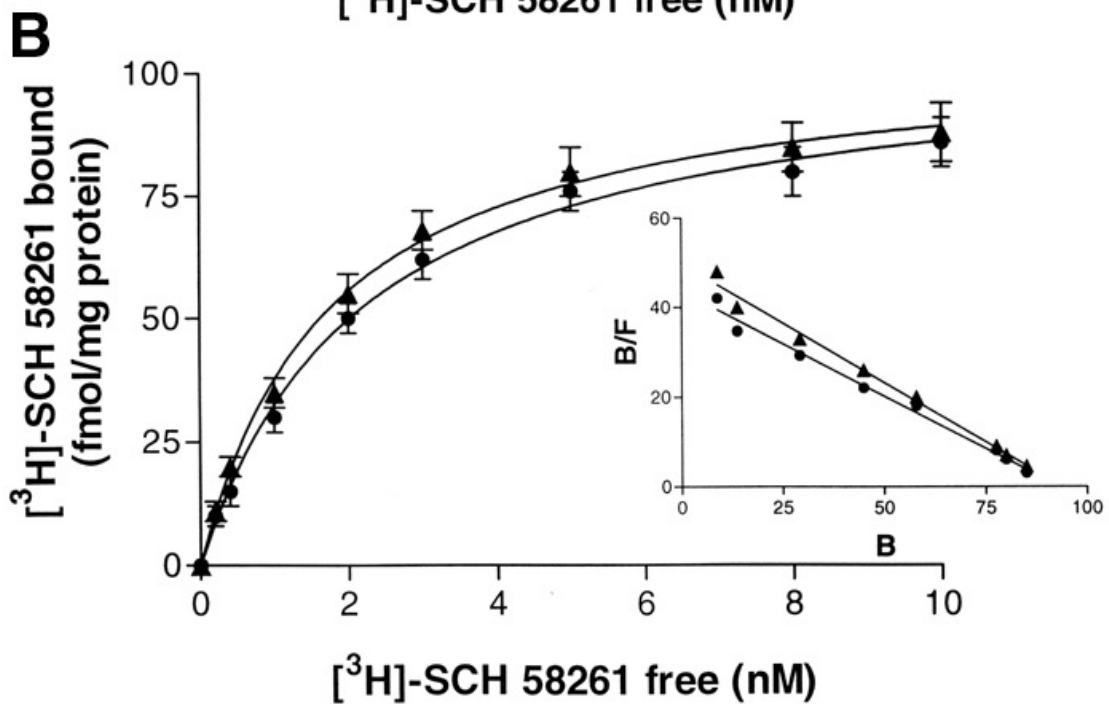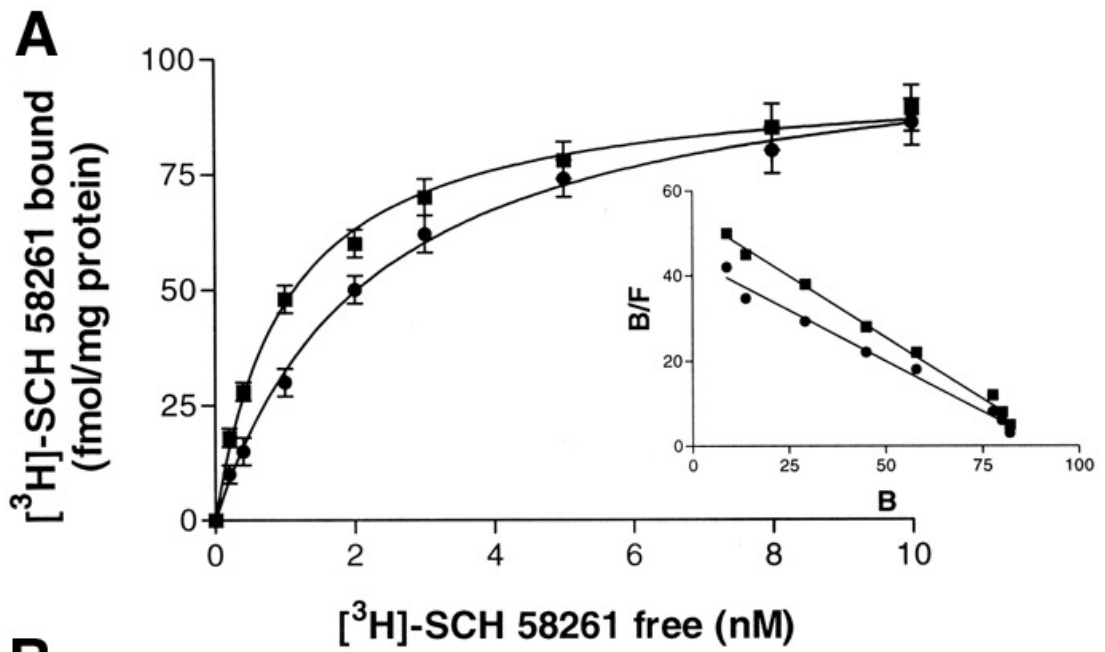
Figure 1.3 - Saturation curves (A) and Lineweaver-Burk analysis (B) of adenylyl cyclase activity stimulated with 3 μM forskolin in ST14A (•) and N548 mu (■) membranes.
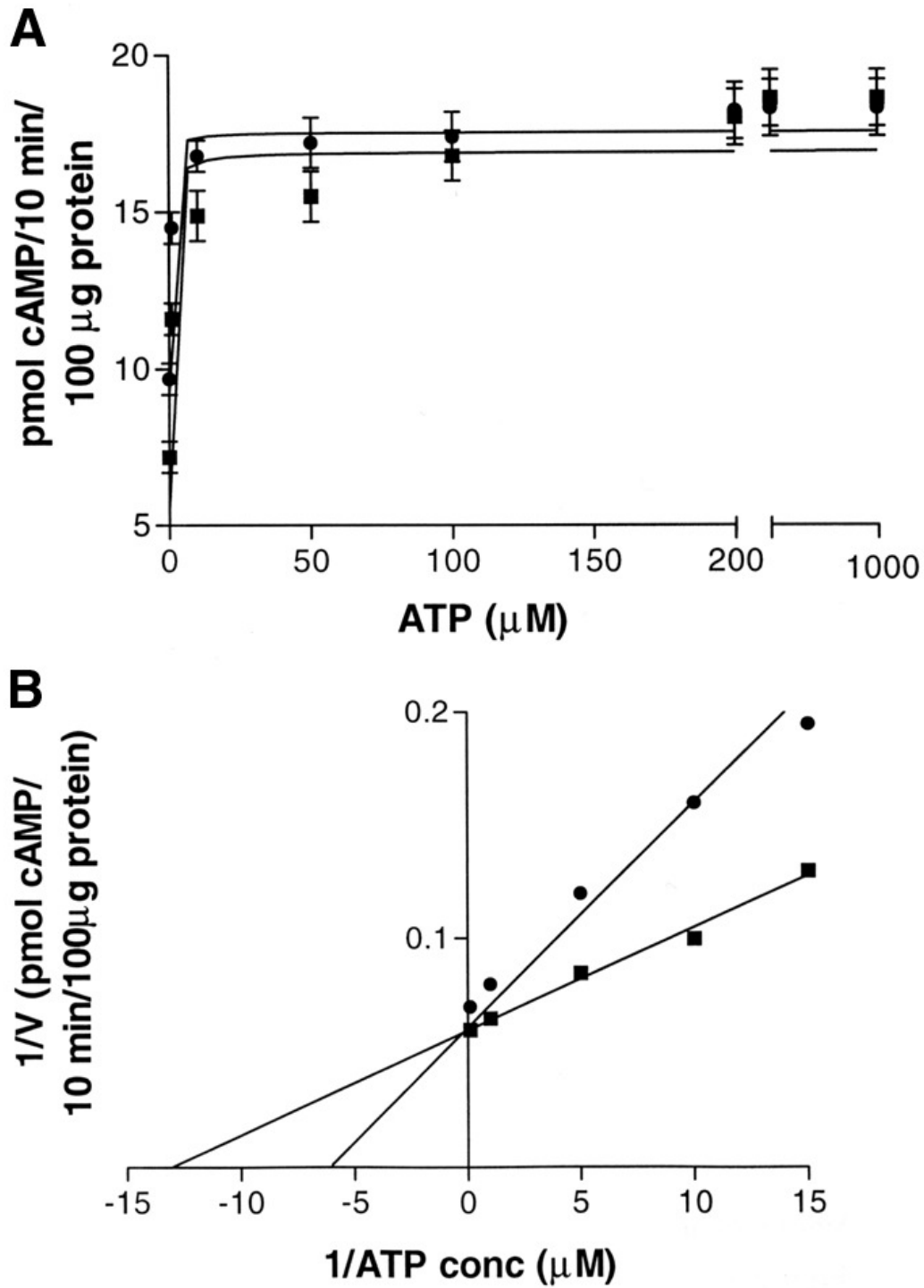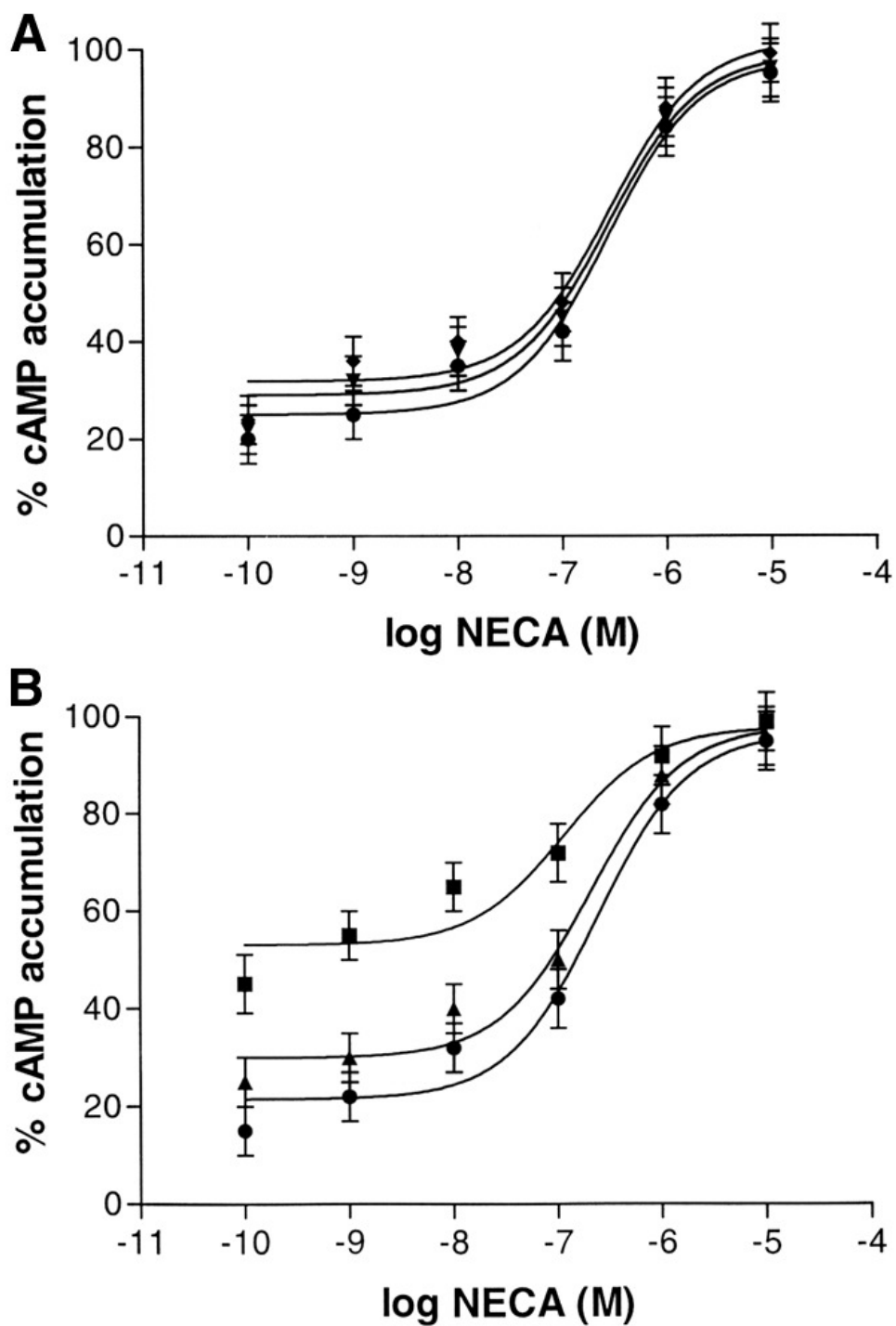
Figure 1.4 - Stimulation of cyclic AMP levels by NECA (0.1 nM – 10 μM) in ST14A (●), in FL wt (▼), in N548 wt (♦) (A), and ST14A (●), in FL mu (▲), in N548 mu (■) (B).

DISCUSSION

The $A_{2A}AR$ plays an important role in striatal function (Ferrè et al., 1993). By activating this receptor subtype in striatal medium spiny neurons, adenosine acts as a dopamine functional antagonist and inhibits the indirect efferent striatal pathway involved in regulation of motor activity. Hence, an intact $A_{2A}AR$ function is needed to preserve an integrated regulation of motor behavior. Besides the involvement of this receptor subtype in motor function, recent data have highlighted its possible role in cell survival, which suggests that its activation under specific pathological conditions may contribute to neurodegeneration in ischemic-like diseases (Abbracchio and Cattabeni, 1999).

The role of the $A_{2A}AR$ in HD is still unclear. Some recent studies have focused on neurotransmitter receptor changes in R6/2 transgenic mice, an animal model of HD in which a fragment of the disease-causing variant of human Htt is expressed (Mangiarini et al., 1996). Reductions of dopamine D_1 and D_2 receptors, $A_{2A}AR$ mRNA, $A_{2A}AR$ binding, and the expression of enkephalin (but not substance P) have been found in these animals (Jacobson et al., 1999). On the contrary, binding autoradiography for glutamate NMDA receptors showed no difference with respect to age-matched control animals, which supports the concept that specific receptor systems may be selectively affected in HD. Moreover, receptor down-regulation seems to appear before the onset of observable symptoms (Bibb et al., 2000). Yet, such changes may still be subsequent to or independent from earlier aberrant receptor(s) behavior. The present study describes, for the first time, an alteration of $A_{2A}AR$ signaling specifically associated with the expression of mutant Htt. Data have been obtained in a cellular system composed of immortalized striatal cells engineered to express either wild-type or mutant Htt. Using this system previously, it was found that Htt protects cells from the action of death triggers via inhibition of the caspases cascade. On the contrary, expression of an

expanded CAG variant of Htt in the same cells led to loss of this protective effect, activation of caspases and apoptosis (Rigamonti et al., 2000).

In the present study it has been shown that both parental striatal cells and the Htt-engineered derivatives express the A_{2A}ARs, as demonstrated by both radioligand binding studies and RTPCR analysis, which hence validates this cellular model as suitable for studying the possible changes induced by mutant Htt on A_{2A}AR function. Consistent with the reduction of A_{2A}AR mRNA in R6/2 transgenic mice (Cha, 2000) is our finding of decreased levels of A_{2A}AR mRNAs in mutant Htt cells. However, the density of the receptor protein was demonstrated to be quite comparable in all clones as detected by radioligand binding studies. Despite no changes of Bmax values, binding studies revealed a moderate but statistically significant increase of affinity in cells expressing mutant Htt, which suggests that mutant, but not wild-type Htt, may interfere with the kinetics of A_{2A}AR binding by endogenous adenosine and may influence the conformation of the ligand recognition site. These results also highlight profound changes of adenylyl cyclase response selectively in mutant Htt cells. Despite no changes of basal adenylyl cyclase activity, response to forskolin was significantly increased in these cells and, more remarkably, an even higher amplification of A_{2A}AR-mediated cAMP formation was observed. Quite surprisingly, direct stimulation of G-protein α-subunit with GTPγS did not result in higher adenylyl cyclase stimulation in mutant Htt cells, which suggests that this subunit is not apparently affected by the mutant protein. This finding may imply a specific effect of mutant Htt on G-protein βγ-subunits, which play key roles in both cyclase activity modulation and in mediating receptor desensitization. A dysfunction of G-protein βγ-subunits leading to reduced receptor desensitization may therefore contribute to the observed aberrant amplification of A_{2A}AR response. From our data it is also clear that changes of A_{2A}AR signaling are much more evident in cells expressing truncated mutant Htt with respect to full-length

mutant Htt. This finding may have intriguing functional implications, because the N548 fragment expressed in these cells mimics one of the potential fragments suggested to be produced by proteolytic cleavage of Htt (Wellington et al., 2000) and which may be required for the expression of mutant Htt cytotoxicity and aggregate formation.

Recent data demonstrate that the full-length mutant protein is processed gradually over-time in vivo to generate amino terminal fragments that accumulate into the nucleus and also in axons and synaptic terminals. Truncated mutant Htt has been also demonstrated to carry significantly higher toxicity with respect to the full-length protein (Wellington et al., 2000). On this basis, one could speculate that N548mu cells may reflect a more advanced stage of the disease, where the aberrant behavior of the $A_{2A}AR$ is maximally expressed. In has been shown that wild-type Htt was protecting cells from death stimuli and mutant Htt was producing cell toxicity (possibly indicating a loss of Htt function by the expanded CAG) (Rigamonti et al., 2000). It is interesting that, in the present report, $A_{2A}AR$ signaling was not modified by wild-type Htt but was selectively altered in mutant Htt cells. It is possible to conclude that, besides loss of the normal protective function of Htt (Rigamonti et al., 2000), the CAG expansion in the mutant protein causes a toxic "gain-of function" activity at the level of the $A_{2A}AR$ signal transduction mechanism, which may result in selective impairment of striatal function, possibly at very initial stages of the disease. A possible role of this receptor in the etiopathology of HD is also consistent with literature suggesting that the $A_{2A}AR$ may contribute to cell death in ischemia-associated neurodegeneration and that $A_{2A}AR$ antagonists may indeed prove useful in preventing such damage (Abbracchio and Cattabeni, 1999). In this respect, our demonstration that selective $A_{2A}AR$ antagonists retain their ability to fully block NECA-stimulated adenylyl cyclase may disclose new avenues in the pharmacological manipulation of Huntington's disease.

CHAPTER 2:

Early and transient alteration of $A_{2A}AR$ signaling in a mouse model of Huntington disease.

INTRODUCTION

Adenosine and dopamine receptors are co-expressed on striatal GABAergic enkephalin neurons. In particular, in these cells, A_{2A}ARs stimulate and D_2 dopamine receptors inhibit adenylyl cyclase and cAMP formation (Ferrè et al., 1993). Adenosine has been also suggested to act as a trigger for development-associated apoptosis and, under specific conditions, activation of the A_{2A}ARs may result in cell death (Jacobson et al., 1999). On this basis, another proposition to explain cell loss in HD is that a dysregulation of adenosine-mediated cell death, likely due to an alteration of A_{2A}AR expression or function, may contribute to neurodegeneration. The role of A_{2A}ARs in cell viability is indeed controversial. Activation of these receptors prevented apoptosis due to serum deprivation in PC12 cells (Huang et al., 2001), and protected sympathetic neurons against nerve growth factor withdrawal, suggesting induction of trophic or protective pathways (Blum et al., 2003). On the other hand, activation of presynaptic A_{2A}ARs on cortico-striatal afferents facilitates release of the excitatory aminoacid glutamate (Popoli et al., 2002), whereas, in glial cells, these receptors might mediate induction of pro-inflammatory enzymes, thus contributing to neuroinflammation (Brambilla et al., 2003). Using A_{2A}AR-/- mice and pharmacological compounds in rats, it was demonstrated that striatal neurodegeneration induced by the mitochondrial toxin 3-nitropropionic acid (3NP, an experimental model of HD) is regulated by A_{2A}ARs. In particular, striatal outcome induced by 3NP depended on a balance between the deleterious activity of presynaptic A_{2A}ARs and the protective activity of post-synaptic A_{2A}ARs (Blum et al., 2003). In line with this dual role of A_{2A}ARs in neurodegeneration both A_{2A}AR agonists and antagonists (Popoli et al., 2002) have been shown to be beneficial in HD. In particular, in R6/2 animals, an established model of HD, treatment with the selective A2A receptor agonist CGS 21680 has been recently reported to

attenuate symptoms (Chou et al., 2005). Interestingly, in the 3NP model of HD, an increase in $A_{2A}AR$ density has been found (Blum et al., 2002).

One of the best characterized animal models of HD is represented by the R6/2 mice (Mangiarini et al., 1996). These animals express exon 1 of the human huntingtin gene with an expanded CAG repeat length of 141-157 under the influence of the human promoter. The R6/2 model has many of the temporal, behavioural and neuropathological features that are observed in HD patients, such as jerky movements and striatal atrophy. At weaning (23 days), R6/2 mice show a lower body and brain weight (Mangiarini et al., 1996). The life span of the affected animals is of 14-15 weeks, with the onset of behavioural phenotype between 8 and 11 weeks. Degeneration within the striatum and non-apoptotic cell death have been observed after 14 weeks (Turmaine et al., 2000), while neuronal atrophy and neuronal intranuclear inclusions have been found at earlier stages (Davies et al., 1997).

On this basis, the present study was undertaken to assess the presence of the aberrant $A_{2A}ARs$ phenotype in the basal ganglia of transgenic R6/2 mice.

MATERIALS AND METHODS

Animals

These studies were performed on heterozygous transgenic R6/2 mice, which express exon 1 of the human huntingtin gene with an expanded CAG repeat length of 141-157, under the influence of the human promoter (Mangiarini et al., 1996).

A R6/2 breeding colony has been established from 15 R6/2 males founders and 35 CBA X C57BL/6F1 (B6CBA) females, all obtained from Jackson Laboratories (Bar Harbor, ME). Transgenic and wild type mice were distinguished by PCR-screening. R6/2 males were mated with B6CBA females to maintain the colony, while R6/2 females were

utilized for the experiments. Non transgenic littermates were used as controls. Measures were taken to minimise pain and discomfort to animals. Mice were killed by decapitation after treatment for 30-45 seconds with CO_2. Tissues for cAMP detection were conserved in ice until assay, while those for RNA extraction or binding studies were frozen immediately after dissection and stored at -80°C.

Experiments have been performed at asymptomatic stage, i.e., postnatal day (PND) 7-8, 9-14, 21, at early symptomatic stages, i.e., postnatal week (PNW) 6 or 8, and at late symptomatic stage, i.e., 11 or 12 PNW.

Genomic DNA extraction

Genomic DNA was extracted at PND 4-5 from the tail of the animals with the NucleoSpin Tissue kit (Macherey Nagel, Germany).

Total RNA extraction

Total RNA was isolated from striata by extraction with Trizol (Invitrogen, US). Reverse transcription of 1,5 µg of RNA was performed with SuperScriptII (Invitrogen, US) according to the manufacturer.

muHtt gene amplification

The amplification protocol has been established on the basis of that reported in the literature (Mangiarini et al.,1996). The amplification of genomic and coding DNA was performed with Taq polymerase DyNAzime (Finnzimes, UK) and specific primers for the human huntingtin gene sequence including the CAG repeat (FW: 5'-GGC GGC TGA GGA AGC TGA GGA-3'; RV:5'- ATG AAG GCC TTC GAG TCC CTC AAG TCC TTC-3').

After denaturation for 3' at 94°C, the amplification was carried out for 35 cycles as follow: 1' at 94°C, 45'' at 65°C, 1' at 72°C. An amplified product of 580 bp corresponding to the expected mw of mutant Htt was detected on a agarose gel 1%.

RT-PCR for A$_{2A}$AR

Qualitative RT-PCR analysis of the A$_{2A}$AR cDNA has been performed using Taq DyNAzyme (Finnzymes, UK) and primers specific for mouse A$_{2A}$AR (FW: 5' – TGT CCT GGT CCT CAC GCA GAG-3'; RV: 5'- CGG ATC CTG TAG GCG TAG ATG AAGG-3'). After denaturation for 3' at 94°C, the amplification was carried out for 35 cycles as follows: 30'' at 94°C, 30'' at 55°C, 1' at 72°C. An expected product of 600 Kb corresponding to the A$_{2A}$AR gene was detected on 1% agarose gels.

Quantitative Real Time PCR for A$_{2A}$AR

Real time quantitative PCR was performed using the SYBR Green kit (Biorad, USA) on a iQ Thermal Cycler (Biorad, USA). The PCR mixtures contain 25 ng of cDNA, 0.2 µM of specific primers and SYBR Green reaction MIX (Biorad, USA). Mixtures were incubated at 95 °C for 3' and then 45 PCR cycles were conducted (95 °C for 30'', 63 °C for 30'' and 72°C for 30''). The sequences of primers are listed below: for A$_{2A}$AR (the target gene), FW: 5'-AAC CTG CAG AAC GTC AC-3' and RV: 5'-GTC ACC AAG CCA TTG TAC CG-3' and for β-Actin (the reference gene), FW: 5'-AGT GTG ACG TTG ACA TCC GTA-3' and RV: 5'-GCC AGA GCA GTA ATC TCC TTC T-3'. Independent Real Time-PCRs were performed using the same cDNA for both the target gene and reference gene. A melting curve was created at the end of the PCR cycle and amplified products were run on 2% agarose gel to confirm that a single product was amplified. Data were analyzed by the iCycler operating software to determine the threshold cycle (CT) above the background for each reaction. The relative transcript

amount of the target gene was normalized to that of β-Actin of the same RNA. Four R6/2 and four control mice have been used at PND 9-14, while three R6/2 and three control mice at the other time points. Except for PNW8, animals are the same utilized for binding studies.

Measurement of adenylyl cyclase activity

Striata were homogenized by 30 strokes with a Teflon glass potter in cold Tris-maleate and DTT buffer pH 7.4, 245 mM and 20 mM respectively.

Incubation medium contained 1 mM ATP, 0.4 mM EGTA, 4 mM $MgCl_2$, 20 µM GTP, 160 mM tris-maleate, 1 I.U./ml adenosine deaminase (Sigma, MO, US), 0.1 mM papaverine, 237 I.U./ml creatine phosphate (Sigma, MO, US) and 310 I.U./ml creatine phosphokinase (Sigma, MO, US). To specifically assess $A_{2A}AR$ mediated stimulation of adenylyl cyclase, graded concentrations of the $A_{2A}AR$ agonist CGS 21680 (Sigma, MO, US) were added to tubes in absence or presence of the $A_{2A}AR$ antagonist ZM 241385 (Sigma, MO, US). Forskolin was also tested at graded concentrations to assess direct adenylyl cyclase stimulation. cAMP levels have been measured in the presence of adenosine deaminase to inactivate endogenous adenosine and in the presence of the phosphodiesterase inhibitor papaverine to prevent cAMP degradation. Each experimental point was repeated at least in triplicate. Samples containing 20 µg protein of homogenate were incubated at 37°C for 15 minutes and then placed at 4°C to stop the reaction. cAMP was extracted by adding cold EtOH 70% for 10 minutes and purified by centrifuging; supernatant was collected and membrane pellet further incubated with EtOH 70%. After 10 minutes samples were centrifuged and supernatant was added to the previously collected one and pellet was discarded. cAMP pellet was then obtained by lyophilization at -80°C. Four transgenic mice and four age- and sex-matched littermates have been used at each time point.

Quantification of cAMP levels

A radioenzymatic assay (Amersham, UK) has been used on cAMP pellets. The assay is based on the competition for a exogenously-added binding protein beetwen [^{3}H]-cAMP and endogenous cAMP. After rehydrating lyophilized pellets in a reaction buffer, a standard amount of [^{3}H]-cAMP was added to samples followed by incubation with the binding protein; unbound cAMP was removed by centrifugation with charcoal. Clear supernatants were then mixed with Formula 999 scintillation liquid (Sigma, MO, US) and counted by using a Beckman liquid scintillation counter. cAMP levels are expressed as pmol/ mg protein/ minutes of incubation with agonist.

Receptor binding assays

Striatal tissues derived from wild type and R6/2 mice were homogenized with a Polytron PTA 10 probe (setting 5, 30 s) in 25 vol of 50 mM Tris HCl buffer, pH 7.4 (Borea et al., 1995). The homogenate was centrifuged at 48000 g for 10 min at 4°C and resuspended in Tris HCl containing 2 I.U./ml adenosine deaminase. After 30 min incubation at 37°C membranes were centrifuged again and used for binding experiments. In saturation binding experiments, striatal membranes were incubated in 50 mM Tris HCl, 10 mM MgCl$_2$ buffer, pH 7.4 with 8 different concentrations of [^{3}H] ZM 241385 ranging from 0.1 to 4 nM. Non specific binding was determined in the presence of 10 μM of ZM 241385. After 60 min of incubation at 4°C, samples were filtered through Whatman GF/B glass fiber filters using a Brandel cell harvester. The filter bound radioactivity was counted on a scintillation counter Tri Carb Packard 2500 TR (efficiency 57%). The protein concentration was determined according to a Bio-Rad method with bovine albumin as reference standard (Bradford, 1976). Saturation binding experiments were analysed with the program LIGAND (Munson and Rodbard, 1980) which performs weighted, non linear least squares curve fitting program. All

experimental data are expressed as mean ± standard error of the mean (SEM) of three or four independent experiments performed in duplicate. Four R6/2 mice at PND 9-14 and three R6/2 at the other time points have been analyzed and compared to an equal number of sex- and age-matched wild type littermates. Except for PNW 6, animals are the same utilized for Real Time PCR.

Statistical analysis

Potency values (EC_{50}) for cAMP production were calculated by non linear regression analysis using the equation for a sigmoid concentration-response curve (GraphPad Prism version 3.03, San Diego, CA, US). Analysis of data was done by 1-way ANOVA. Differences between wild type and R6/2 mouses were analyzed with Student's t test. Differences were considered significant at a value of $p<0.01$. All data are reported as mean ± SEM of three or four independent experiments.

RESULTS

Expression of mutated human huntingtin and $A_{2A}ARs$ in striatum of R6/2 mice was confirmed by qualitative RT-PCR at every age (data not shown).

To assess the receptor activity, we performed ex-vivo measurements of cAMP production in homogenates from striata in the absence (basal activity) or presence of the selective $A_{2A}AR$ agonist CGS 21680, or after direct activation of the enzyme with forskolin, both utilized at graded concentrations. A time course analysis of $A_{2A}AR$-dependent adenylyl cyclase activity has been performed at various presymptomatic and symptomatic stages. On the basis of results, 3 early presymptomatic ages (PND 7-8, 9-14 and 21), 1 early symptomatic stage (PNW 8) and 1 late symptomatic age (PNW 11) are reported here. Potency values (EC_{50}) of CGS 21680 and forskolin on cAMP production were calculated by non linear regression analysis using the equation for a

sigmoid concentration-response curve and by one-way ANOVA. At any age considered, basal cAMP values were not statistically different in R6/2 and wild type mice (e.g., at PND 7-8, levels in pmol/min/mg prot were $10.6 + 0.12$ and $10.3 + 0.14$ in wild type and R6/2 animals, respectively; at all other ages, highly similar levels were detected). As expected, at all developmental stages, CGS 21680 concentration-dependently increased adenylyl cyclase activity in both wild type and R6/2 mice (Fig. 2.1, Table 2.1). At PND 7-8, a supersensitivity of $A_{2A}AR$-dependent adenylyl cyclase activity was found in R6/2 with respect to wild type mice, as demonstrated by a left-ward shift of CGS21680 concentration-response curve and a significant reduction of agonist EC_{50} value (Fig. 2.1, Table 2.1). At PND 9-14, the increase of $A_{2A}AR$-mediated adenylyl cyclase responsiveness in R6/2 mice was even more marked (Fig. 2.1), as demonstrated by an EC_{50} value of the $A_{2A}AR$ agonist CGS 21680 ten fold lower with respect to corresponding EC_{50} value in wild type mice ($175+23$ nM, respect to $1922+197$ nM) (Table 2.1). $A_{2A}AR$-dependent adenylyl cyclase activity in R6/2 mice was back to control levels at PND 21 (Fig. 2.1, Table 2.1) and remained comparable in wild type and R6/2 mice at PNW 8 and PNW11 (Fig. 2.1, Table 2.1). In a similar way, potency of forskolin in stimulating adenylyl cyclase activity was enhanced in R6/2 with respect to wild type mice at PND 7-8 and 9-14, as shown by significantly lower EC_{50} values on cAMP production (Table 2.2). No significant differences of forskolin-stimulated adenylyl cyclase activity were observed in R6/2 mice with respect to wild type animals at later developmental ages (i.e., PND21 and PNW11, Table 2.2). We then studied the effect of the selective $A_{2A}AR$ antagonist ZM 241385 on $A_{2A}AR$-stimulated adenylyl cyclase in these animals. Antagonism of agonist-stimulated adenylyl cyclase in both wild type and R6/2 mice was found at all the stages, except for PND 9-14 (Fig. 2.2). At this developmental age, when the aberrant $A_{2A}AR$ phenotype is most evident in R6/2 mice (Fig. 2.1), the ability of ZM 241385 to selectively counteract $A_{2A}ARs$-mediated

104

cAMP production was markedly impaired with respect to control mice (Fig. 2.2). In fact, in R6/2 mice at this developmental stage, cAMP levels measured in the presence of both CGS 21680 and ZM 241385 were not significantly different with respect to CGS 21680 alone (Fig. 2.2). Conversely, at PND 21, when in transgenic mice adenylyl cyclase responsiveness is back to control levels (Fig. 2.1), the ability of ZM 241385 to counteract CGS 21680- induced cAMP production was fully restored (Fig. 2.2).

Furthermore, in order to investigate if the increase in cAMP production observed at early stages is related to changes in $A_{2A}AR$ expression or density, quantitative Real Time PCR and binding studies have been performed. Real Time PCR analysis at PND 9-14, PND 21, PNW 8 and PNW 12 revealed no changes at PND 9-14, and decreased expression starting from PND 21 in R6/2 mice in comparison with wild type animals (Fig. 2.3).

Binding studies with the antagonist radioligand [^{3}H] ZM 241385 at PND 9-14, PND 21, PNW 6 and PNW 12 revealed a significant increase in receptor density (Bmax) at PND 9-14 with no changes in affinity (K_D) (Table 2.3; Fig. 2.4); no differences in either receptor affinity or density were detected at later times (Table 2.3; Fig. 2.4). Except for PNW6 and PNW 8, the striata utilized for binding activity and Real Time PCR were from the same animals.

Table 2.1. EC$_{50}$ values of CGS 21680 on cAMP stimulation in striatal membranes from wild type and R6/2 mice.

	Wild type mice	*R6/2 mice*
PND 7-8	2020 ± 185	$589 \pm 67*$
PND 9-14	1922 ± 197	$175 \pm 23*$
PND 21	2051 ± 208	2046 ± 204
PNW 8	1983 ± 206	2180 ± 189
PNW 11	1977 ± 178	2032 ± 215

*, P<0.01 vs wild type mice.

Table 2.2 - EC_{50} **values of Forskolin on cAMP stimulation in striatal membranes from wild type and R6/2 mice.**

	Wild type mice	*R6/2 mice*
PND 7-8	956 ± 72	540 ± 47*
PND 9-14	1120 ± 115	267 ± 28*
PND 21	1060 ± 103	881 ± 84
PNW 11	845 ± 90	847 ± 87

*, $P < 0.01$ vs wild type mice.

Table 2.3 - Binding parameters of A_{2A}ARs on striatal membranes from wild type and R6/2 mice.

	Wild type mice	*R6/2 mice*
PND 9-14	$K_D=0.20\pm0.02$ Bmax=1083±19	$K_D=0.19\pm0.02$ Bmax=1376±22*
PND 21	$K_D=0.22\pm0.04$ Bmax=1107±41	$K_D=0.21\pm0.03$ Bmax=988±22
PNW 6	$K_D=0.21\pm0.01$ Bmax=1104±39	$K_D=0.21\pm0.02$ Bmax=975±32
PNW 12	$K_D=0.21\pm0.02$ Bmax=1125±46	$K_D=0.20\pm0.02$ Bmax=967±38

*, $P<0.01$ vs the other wild type mice and vs R6/2 mice of PND 21 and PNW 6 and 12.

Figure 2.1 - A$_{2A}$AR-stimulated adenylyl cyclase activity in wild type (■) and R6/2 mice (●) of various ages in presence of graded concentrations of the A$_{2A}$AR agonist CGS 21680.
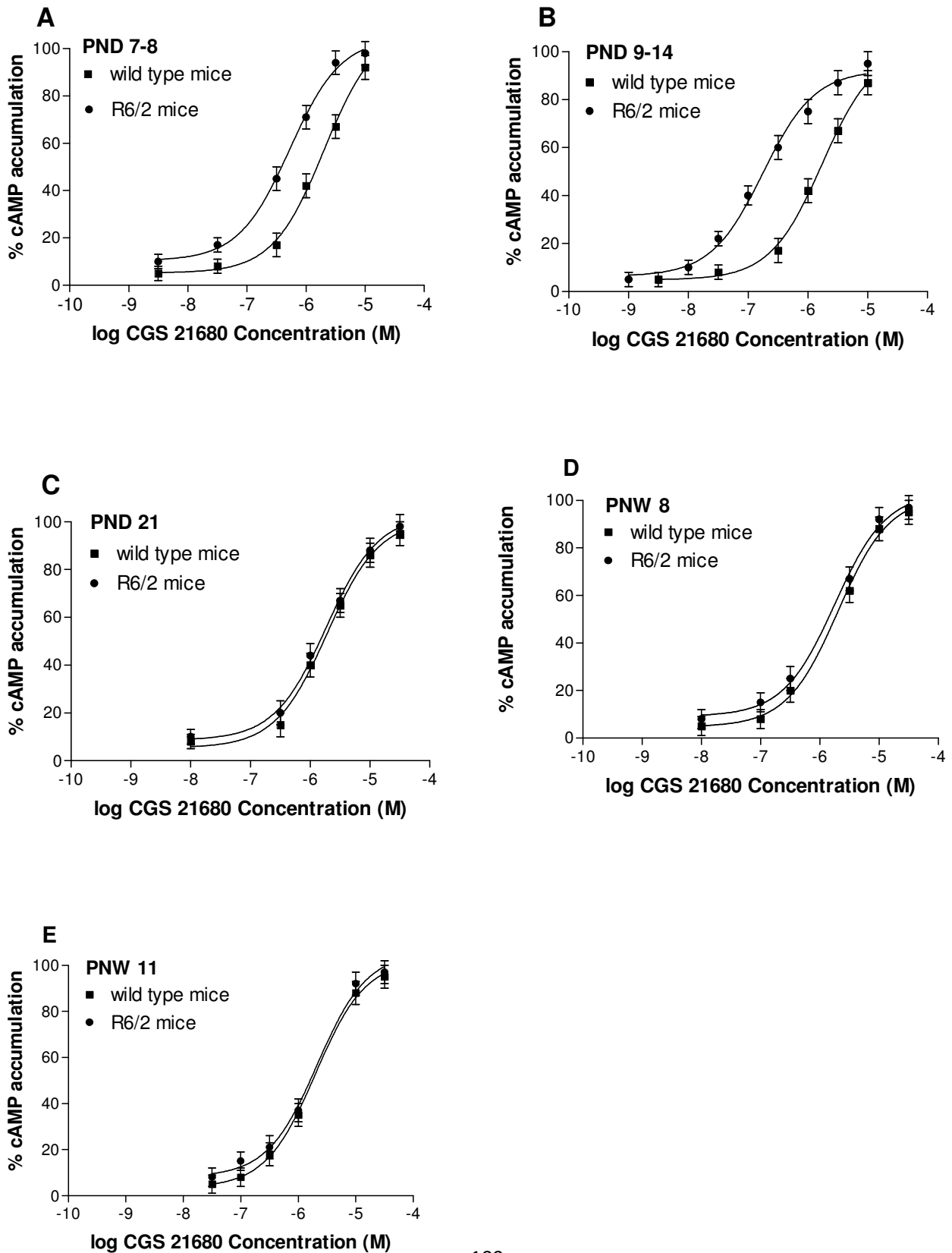
Figure 2.2 - Effect of the selective $A_{2A}AR$ antagonist ZM 241385 on $A_{2A}AR$-mediated cAMP production. The ability of antagonist to counteract cAMP production was assessed against 0.5 µM CGS 21680 in wild type (wt) and R6/2 mice as indicated.
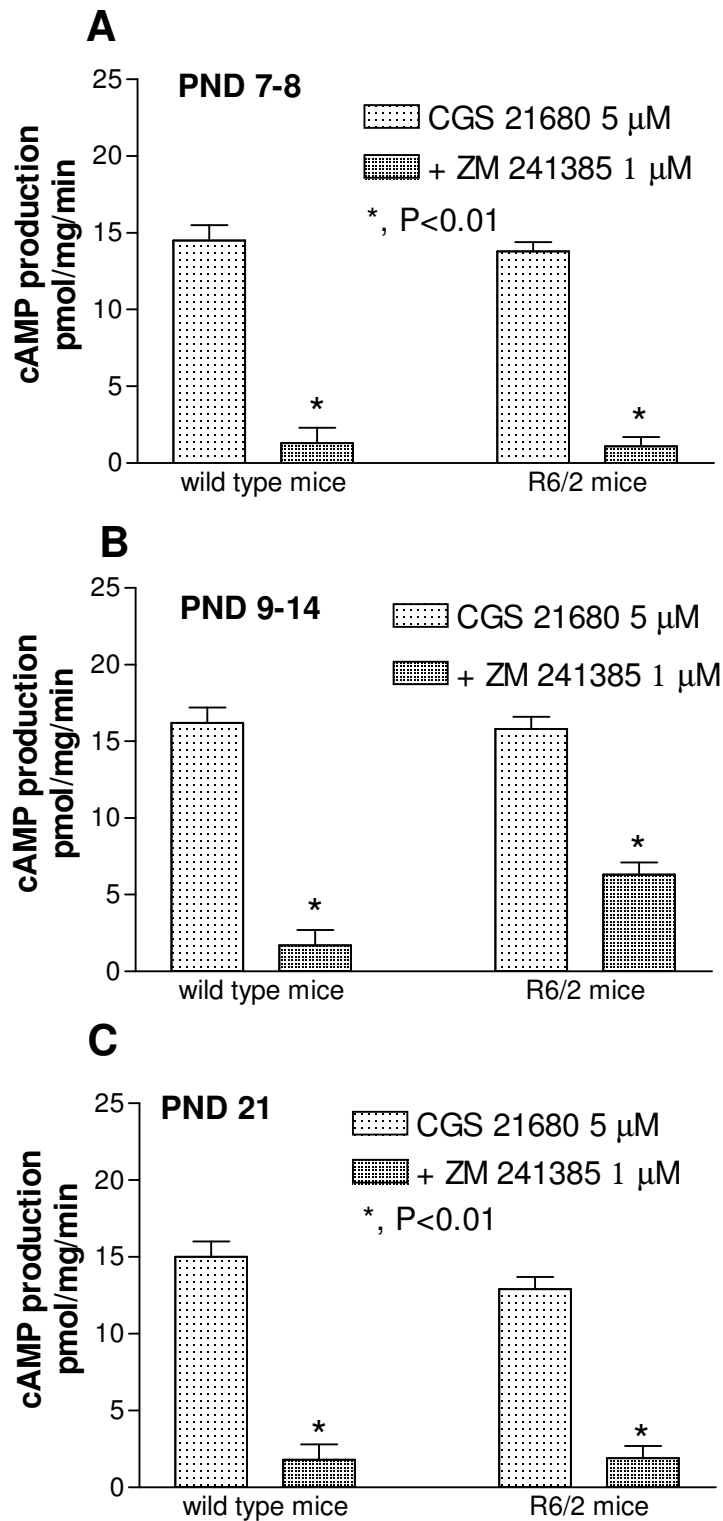
Figure 2.3 - Real Time PCR analysis of $A_{2A}AR$ expression in wild type (open bars) and R6/2 mice (filled bars) at different ages. At each time point, receptor expression value in R6/2 mice is reported as % of correspondent value in wild type animals set as 100%. *, P < 0.01 vs controls.
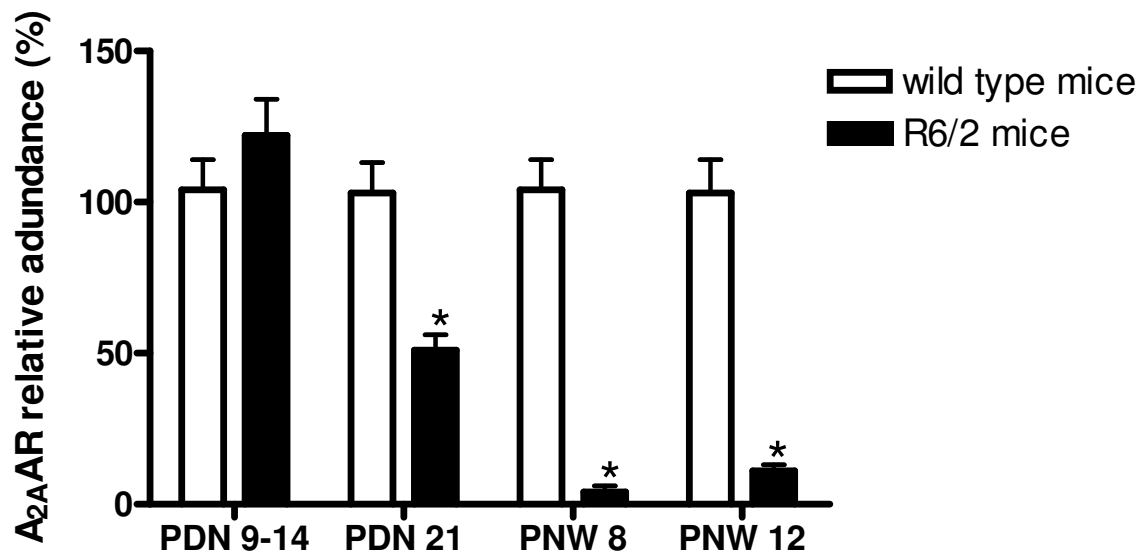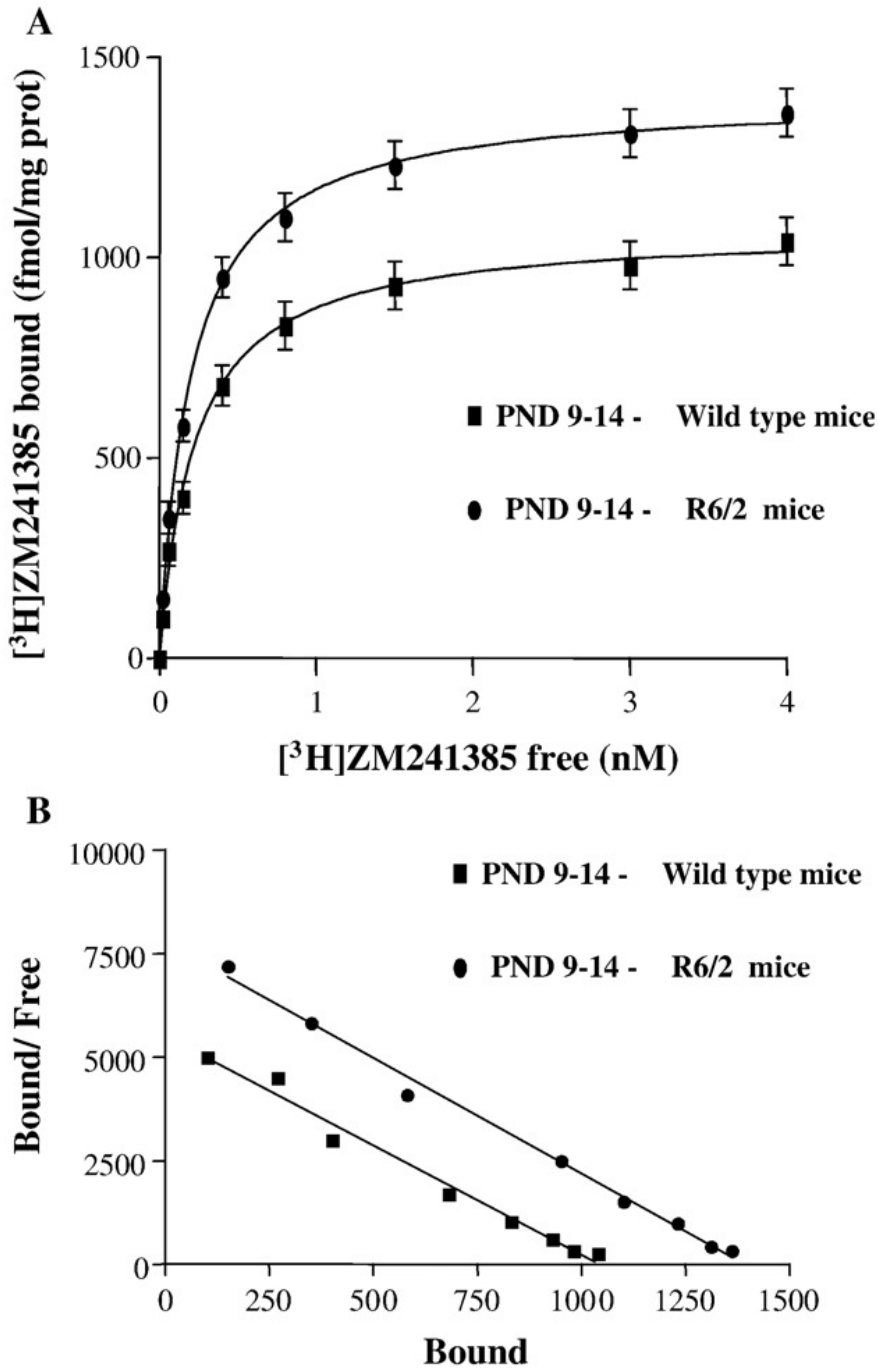
Figure 2.4 - A: Saturation curves for [³H] ZM 241385 binding to A$_{2A}$ARs in striatal membranes from wild type and R6/2 mice at PND 9-14. **B:** Scatchard plot analysis of the same data reported in **A.**

DISCUSSION

In the present study, we demonstrate that R6/2 mice exhibit a transient, but significant, increase of $A_{2A}AR$ density (Bmax) and $A_{2A}AR$ -stimulated adenylyl cyclase activity in striatum, one of the brain areas most heavily affected in HD. We speculate that the increased receptor number in R6/2 mice might be at the basis of the detected increase of $A_{2A}AR$-dependent adenylyl cyclase. This aberrant $A_{2A}AR$ phenotype is maximally expressed well before the onset of motor symptoms, i.e., between PND 7 and 14, and, at PND 9-14, is accompanied by a transient insensitivity of adenosine $A_{2A}ARs$ to selective antagonists such as ZM 241385. The reasons at the basis of this insensitivity are currently unknown, but it may be hypothesized that, at PND 9-14, an alteration of receptor conformation might impair the ability of the antagonist to counteract agonist-induced receptor activation. The aberrant $A_{2A}AR$ phenotype disappears at PND 21 (when, in R6/2 mice, sensitivity of $A_{2A}AR$ -dependent adenylyl cyclase to the selective antagonist ZM 241385 is also restored, and receptor density returns to values similar to wild type mice). Forskolin-stimulated adenylyl cyclase activity was also increased in the R6/2 animals at PND 7-8 and (more evident) at PND 9-14.

Despite increased receptor density in radioligand binding studies, at PND 9-14, by employing Real Time PCR, we did not find any significant change of $A_{2A}AR$ mRNA in R6/2 mice with respect to wild-type animals. This suggests that, at this developmental age, receptor turn-over rather than receptor synthesis may be altered in these animals. Starting from PND 21, in R6/2 mice, receptor density is back to control values, as assessed by binding studies; instead, at this and at later ages, dramatic, time-dependent decreases of $A_{2A}AR$ mRNA are found in transgenic mice with respect to wild-type animals. These data are not due to variability between animals, since, with the only exception of PNW 6 or 8, at all other ages binding and expression data have been obtained on striata from the same animals. Our data indicating decreased $A_{2A}AR$

synthesis in HD animals approaching the symptomatic stage are in line with a recent study showing that, in PC12 cells and primary striatal neurons, expression of mutant Htt with expanded poly(Q) significantly reduced the transcript levels of the endogenous $A_{2A}AR$ (Chiang et al., 2005). In our study, the apparent discrepancy between binding and expression data (i.e., no change of receptor density in cell membranes despite dramatic decreases of receptor mRNA) are indicative of a reduction of receptor turn-over in transgenic mice. This may indeed represent a compensatory mechanism by which cells respond to decreased receptor synthesis.

The increase of $A_{2A}AR$ mediated- and forskolin-stimulated adenylyl cyclase in the R6/2 mice is consistent with our previous demonstration of an aberrantly-increased $A_{2A}AR$ phenotype in HD. Importantly, this is one of the first signs of mutant huntingtin toxicity in R6/2 mice, which precedes of 4 weeks the previously demonstrated change in responsiveness to glutamate (Cepeda et al., 2001). The transitory nature of this dysfunction supports the hypothesis of compensatory mechanism that may mask an early functional abnormality, pushing symptoms recognition towards more advanced stages.

The precise mechanism by which mutant huntingtin causes HD is still unknown. Both gain-of-function alterations in which mutant huntingtin has protein level toxicity, as well as loss of function of normal huntingtin have been proposed to contribute to HD (Beal and Ferrante, 2004). Proteolysis of mutant huntingtin with formation of toxic protein aggregates in the nucleus, cytoplasm and processes of neurons may represent a primary event contributing to early neuropathology. This also triggers cascades of both damaging and compensatory molecular processes and genetic programs leading to increasingly dysfunctional neurons that are susceptible to more generic stresses. These stresses include oxidative injury, transcriptional dysregulation, increased activity of excitotoxic glutamate receptors (Li et al., 2003), expression of inflammatory and pro-

apoptotic signals, malfunctioning proteolysis, increased transglutaminase activity, mitochondrial dysfunction and energy depletion (Beal and Ferrante, 2004). These changes are accompanied by neurochemical alterations involving glutamate, dopaminergic and adenosine receptors (Pavese et al., 2003). In this respect, the aberrant $A_{2A}AR$ behaviour in cells expressing mutant huntingtin and in the striatum of HD R6/2 mice together with the selective expression of $A_{2A}ARs$ in the population of striatal neurons that degenerate early in HD (Glass et al., 2000), suggest that a dysfunction of $A_{2A}AR$ may be related to the underlying disease mechanism. Surprisingly, the early change of $A_{2A}AR$ signalling detected in this study is apparently compensated in adult animals; however, it is not uncommon that early insults during development seem to be initially compensated, but indeed accumulate with other types of damage leading to cytotoxicity later during life. In an electrophysiological study performed on the same animal model, a marked and transient increase in large-amplitude synaptic spontaneus events in striatal medium-spiny neurons was found at 5-7 weeks of age (Cepeda et al., 2003). These excitatory post-synaptic currents (EPSC) primarily represent the effects of spontaneous excitatory neurotransmitter (e.g., glutamate) release in the striatum, and are taken as an indicator of corticostriatal integrity. At a later age (11 weeks), the frequency of these events in R6/2 mice did not statistically differ with respect to wild type animals. This alteration of the cortico-striatal pathway highlights a transient change of glutamatergic inputs that are likely to contribute to the later vulnerability of striatal neuron to degeneration (Cepeda et al., 2003).

Overall, these findings seem to place the striatal $A_{2A}AR$ among those subtle early events that may be involved in the disease process, but appear temporarily compensated. If this is the case and considering the large degree of attention in the development of compounds that modulate $A_{2A}AR$ activities, exposure to these compounds at very early presymptomatic stage should not remain unexplored.

CHAPTER 3:

Aberrant A_{2A}AR expression and function in peripheral blood cells of patients with Huntington's disease.

INTRODUCTION

Although no cure is currently available, recent advancements in the understanding of the molecular events underlying HD are expected to disclose novel pharmacological and neurosurgical approaches in the years to come. In this context and given the progressive manifestation of the disease, the identification of peripheral biomarkers of HD is also of crucial importance. Potentially, an ideal biomarker of HD should (i) be easily accessible and measurable in peripheral cells, (ii) show limited or no variability within the control population; and (iii) should reflect stages or a state (intensity) of degeneration. Various studies indicate that membrane receptors present in neurons of the central nervous system (CNS) may also be expressed in human peripheral blood cells, which, when studied in patients with CNS pathologies, exhibit alterations similar to those observed in postmortem brain tissues or in genetic animal models of the diseases (Cha et al., 1999; Varani et al., 1999). In this respect, a particularly interesting target to be investigated in HD is represented by the $A_{2A}AR$.

On this basis, the present study was undertaken to assess the status of the $A_{2A}AR$ in peripheral blood cells of HD-affected and non-affected mutation carrier subjects compared with control healthy individuals.

Two strategies were adopted to assess the status of the $A_{2A}AR$ in peripheral cells from HD subjects. The first one aimed at measuring the binding kinetic parameters of the $A_{2A}AR$ receptor ligand [^{3}H]-ZM 241385 in platelets, lymphocytes and neutrophils of patients with Huntington disease and non-affected mutation carrier subjects compared with control subjects. The second one aimed at assessing the ability of $A_{2A}AR$ agonists to stimulate adenylyl cyclase activity and cAMP formation in the blood cells of the three group of subjects.

MATERIALS AND METHODS

Patients

For this study, we enrolled a cohort of 48 heterozygous (22 males, 26 females) and 3 homozygous unrelated patients (1 male, 2 females) (Table 3.1). All patients whose data have been collected within a databank since 1997 (Squitieri et al., 2001) were seen and longitudinally monitored by senior neurologists with expertise in the field. The onset of the disease was defined as the time when either motor clinical manifestations (i.e., choreic movements or other extrapyramidal symptoms) or severe psychiatric symptoms changing the normal life state, first became noticeable (Squitieri et al., 2003). Table 3.1 shows the mean age of heterozygous patients and asymptomatic mutation carriers. For the three homozygous patients, the mean age was 60.7 ± 5.8 years. Motor symptoms and behavioral changes were assessed with the Unified Huntington's Disease Rating Scale (UHDRS; 14). Most patients were treated with low doses of neuroleptics or benzodiazepines. The 48 heterozygous patients enrolled were matched for similar age to the cohort of control subjects and were divided into three groups: 39 by age in years 45.7 (± 2.1) (Table 3.1) were analyzed for $A_{2A}AR$ activity (Table 3.1). The same parameters were measured in five additional patients who had never been treated with either neuroleptics or benzodiazepines. Finally, four other patients were subsequently enrolled for the measurement of non-$A_{2A}AR$ subtypes (Table 3.2).

Mutation carriers at the pre-symptomatic stage

Seven at-risk subjects (5 males and 2 females) were informed of their increased risk of developing HD after performing predictive testing (PT). For the study, among the at-risk subjects who underwent the PT (15), we selected those with no evidence of even minor neurological signs or soft motor symptoms. To do that, we performed careful neurological exam by UHDRS, MRI, and additional cognitive assessments, other than

those included in UHDRS, specifically the Wechsler Assessment Intelligence Scale (WAIS) and the Mini Mental State Examination. The cognitive and behavioral assessments were re-evaluated after six months in an attempt to look at even subtle initial non-motor pathological changes of HD in these subjects. By this strategy, we selected mutation carrier subjects in a pre-symptomatic life stage, far enough from the beginning of HD. This explains why the age of at-risk subjects is younger than that of the already affected HD subjects (Table 3.1).

Healthy control subjects

The protocol was approved by the local Ethics Committee, and written informed consent was obtained from each participant in accordance with the principles outlined in the Declaration of Helsinki. For the controls, 58 healthy subjects (28 males and 30 females) were included in the study (Table 3.1). In several cases, healthy controls were volunteers from Ferrara University Hospital Blood Bank. In other instances, subjects with no mutation were enrolled among either the unaffected spouses or the patients admitted to the hospital at Neuromed whose diagnosis was other than neurodegenerative disease or cardiovascular pathology (i.e., patients admitted in the Neurosurgery Department). After careful retrospective disease history, all the subjects with suspected neurological, psychiatric (i.e., behavioral disturbances), and cardiovascular pathologies were excluded from the study. All healthy subjects were unrelated to the mutation carriers. The 58 controls were matched for similar age to the cohort of HD subjects and were divided into three groups: 44 by age in years 46.6 (±1.8), 10 by age in years 31.1 (1.5) (Table 3.1), and four subjects who were subsequently enrolled for the measurements of non-$A_{2A}AR$ receptor subtypes (Table 3.2).

Ethical approval

For this project, we obtained a formal ethical approval by the Bioethics Committee according to the Helsinki Declaration and as also required by Telethon Foundation, before performing the study. An informed consent, specific for the study, was required from each subject. For PT, a different informed consent is obtained from at-risk subjects enrolled in the presymptomatic program at Neuromed. This program was also formally approved by the Bioethics Committee. After the subjects' consent, a blood sample was also collected for general investigation.

Mutation study

DNA was extracted from blood lymphocytes. Polymerase chain reaction was used according to the published methods to study CAG trinucleotide repeats and the close CCG polymorphism (Squitieri et al., 1994). Reference samples and standardized sequence clones for sizing are used for the genetic test. All HD subjects had a CAG repeat number in the pathological range (mean expanded number of 44.9±5.4 CAG repeats, range 39–70 CAG). Normal controls showed a CAG repeat number within the normal range (18.4 ± 4.2, range 15–24).

$A_{2A}AR$ receptor study: preparation of peripheral blood cells or membrane suspensions

Peripheral venous blood samples were obtained from HD subjects and healthy controls. The isolation of the various cell fractions (platelets, lymphocytes, neutrophils) started no later than 6–8 hours after the samples had been taken. Membranes from human platelets, lymphocytes, and neutrophils were prepared as previously described (Varani et al., 1997;1998) and used for radioligand binding assays. Washed platelets, lymphocytes, and neutrophils were prepared to evaluate cAMP levels (Varani et al.,

1997;1998). Two strategies were adopted to assess the status of the $A_{2A}AR$ in peripheral cells from HD subjects. The first one aimed at measuring the binding kinetic parameters of the $A_{2A}AR$ ligand [3H]-ZM 241385 compared with control subjects. The second one aimed at assessing the ability of $A_{2A}AR$ agonists to stimulate adenylyl cyclase activity and cAMP formation. Due to the larger volume of blood necessary for the cAMP assay, the latter has been done in a lower number of patients with respect to the radioligand binding study.

[3H]-ZM241385 binding assay to $A_{2A}ARs$ on membranes from peripheral blood cells

Saturation binding experiments were performed by incubating membranes (50 µg of protein per assay) from the different cellular fractions with 8–10 concentrations of the $A_{2A}AR$ antagonist ([3H]- 4- (2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]-ethyl)phenol) [3H]-ZM 241385 (0.01–10 nM) in a total volume of 250 µl containing 50 mM Tris HCl buffer and 10 mM $MgCl_2$, pH 7.4. The incubation time was 60 min at 4°C to allow the equilibrium to be reached. Non-specific binding was determined in the presence of N-ethylcarboxamido-adenosine (NECA) 10 µM and was about 25% of total binding. Bound and free radioactivities were separated by filtering the assay mixture through Whatman GF/C glass fiber filters by use of a Brandel cell harvester. The filter-bound radioactivity was counted by using a Beckman liquid scintillation counter with an efficiency of 55%. [3H] DPCPX and [3H] MRE 3008F20 binding assays to A_1ARs and A_3ARs on membranes from peripheral blood cells.

Binding to A_1ARs adenosine receptors was performed with [3H] DPCPX (0.2–20 nM) in membranes obtained from human lymphocytes in a 50 mM Tris HCl buffer, pH 7.4, for 120 min at 4°C, following the same technical procedure used for $A_{2A}AR$ binding. Non-specific binding was determined with 100 µM of NECA (Varani et al., 2000).

Binding to A_3ARs was performed by incubating membranes obtained from human neutrophils with [^{3}H] MRE 3008F20 (0.2–20 nM) in a 50 mM Tris HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ and 1 mM EDTA for 120 min at 4°C. Non-specific binding was determined with 1 μM of MRE 3008F20 (Varani et al., 2000). [^{3}H] UK 14304 binding assays to α2-adrenergic receptors on membranes from blood platelets. Binding to α2-adrenergic receptors on human platelets was performed with [^{3}H] UK 14304 (0.2–20 nM) in a 50 mM Tris HCl buffer, pH 7.4, containing $MgCl_2$ 10 mM for 60 min at 25°C, and following the same technical procedure used for $A_{2A}AR$ binding. Non-specific binding was determined with 10 μM of UK 14304 (Varani et al., 1999).

Measurement of cyclic AMP levels in peripheral blood cells

Adenylyl cyclase activity was evaluated by incubating washed peripheral blood cells (either platelets or lymphocytes or neutrophils) in the absence or presence of 6–8 different concentrations of the $A_{2A}AR$ agonist NECA. Cells were suspended in 0.5 ml of buffer containing 2 I.U. of adenosine deaminase and 0.5 mM 4-3-butoxy-4-methoxybenzyl-2-imidazolidinone (Ro 20-1724) as a phosphodiesterase inhibitor and were preincubated in a shaking bath at 37°C. NECA was added to the mixture and the incubation continued for another 10 min. The reaction was terminated by addition of cold 6% trichloroacetic acid (TCA). The TCA suspension was centrifuged at 2000 x g for 10 min at 4°C, and the supernatant was extracted four times with water-saturated diethyl ether. The final aqueous solution was tested for cAMP levels by a competition protein-binding assay. Samples of cAMP standards (0–10 pmol) were added to each test tube containing the binding protein (previously prepared from beef adrenals and incubated at 4°C for 150 min). After addition of charcoal, the samples were centrifuged at 2000 x g for 10 min. The clear supernatant was mixed with Atomlight (Perkin-Elmer, Beltsville, MD) and counted by using a Beckman liquid scintillation counter.

Statistical analysis

A weighted nonlinear least-squares curve-fitting program LIGAND (Munson and Rodbard, 1980) was used for computer analysis of the data from the binding saturation experiments, as previously described. EC_{50} values in the cAMP assay were calculated with the non-linear least-squares curve-fitting program Prism (Graph PAD, San Diego, CA). Nonparametric tests (Mann Whitney U) were used to compare each class of HD subjects (affected heterozygotes, presymptomatic heterozygotes, and homozygous patients) and healthy controls. Differences were considered significant at a value of $P < 0.01$. All data are reported as mean ± SEM.

RESULTS

Figure 3.1 shows [^{3}H]-ZM 241385 saturation curve in membranes prepared from platelets, lymphocytes, and neutrophils from control and HD heterozygous subjects. In all cell preparations from control subjects, $A_{2A}AR$ binding was saturable and indicated the presence of a single receptor population characterized by affinity (expressed as ligand dissociation constant, K_D) and density (expressed as femtomoles of bound ligand per milligram of protein, Bmax) values that are in agreement with those previously reported in the literature for these human cell populations (Table 3.1; Varani et al., 2003). In particular, and as expected, this analysis in control healthy subjects revealed a uniform level of $A_{2A}ARs$ in the peripheral blood cells of all the subjects. In only two cases, which have been nevertheless included in the data analysis of the control group, increased $A_{2A}AR$ values were present (the reasons at the basis of this deviance from the other control values are presently unknown). Analysis of the same parameters in HD subjects revealed a statistically significant increase in $A_{2A}ARs$ density (Bmax) in all cell populations with respect to controls (P=0.0001 versus control, Table 3.1, Fig. 3.1). A

statistically significant difference was obtained by considering either the HD subjects as a whole (HD patients and at risk mutation carriers) or each cohort of mutation carriers compared to healthy controls (heterozygotes $P<0.01$, asymptomatic at risk $P<0.0001$, Table 3.1). The increase in Bmax was accompanied by an increase in K_D values ($P<0.0001$ vs. control, Table 3.1). Also in this case the difference was highly significant when considering either the whole cohort of mutation carriers ($P<0.01$) or each cohort of HD subjects ($P<0.01$, Table 3.1). Given that the mean age of asymptomatic mutation carriers is lower than that of symptomatic patients, 10 additional age-matched healthy subjects (5 males and 5 females) were included in the protocol and subsequently analyzed (Table 3.1). As shown in Table 3.1, this analysis revealed $A_{2A}AR$ parameters that were in agreement with those obtained from the samples from the other control subjects. This indicates that receptor changes in the at-risk mutation carriers are related to their genetic status (Table 3.1).

Subsequent analysis of $A_{2A}AR$ K_D and Bmax values in three subjects homozygous for the HD mutation also revealed highly increased $A_{2A}AR$ parameters in all three blood cell types compared with controls (Table 3.1). In spite of the limited number of blood samples from homozygous patients available, neutrophil cells from these subjects showed even significantly higher ligand dissociation constant (K_D) and Bmax with respect to heterozygotes (Table 3.1). Finally, in all groups considered, the values of K_D and Bmax did not seem to correlate to the triplet number and age at onset, and neither seemed to depend on the number of HD years. Because both the density and the functional response of $A_{2A}ARs$ may be influenced by drugs, such as neuroleptics and/or benzodiazepines that are generally administered to these patients (Parson et al., 1995), we have performed a study where we measured $A_{2A}AR$ -binding parameters in the lymphocytes of a small cohort of 5 HD affected patients who had never been treated with these drugs. In these patients, a K_D value of 3.54 ± 0.35 nM and a Bmax value of

223 ± 9 fmol/mg protein were found, which were highly statistically different with respect to the corresponding values in control subjects (P<0.01). Furthermore they did not differ from the binding parameters obtained in the other HD patients included in the present study. These results suggest that the detected $A_{2A}AR$ phenotype correlates with the genetic status of the patients and is unlikely to be affected by pharmacological treatments generally administered to these patients. Moreover, to verify whether the observed changes in $A_{2A}AR$ activities are specific for this receptor subtype or also involve other types of membrane GPCRs, we have evaluated the binding parameters (K_D and Bmax) of two additional adenosine receptors (the A_1AR and A_3AR subtypes), and of another GPCR unrelated to this receptor family (i.e., the α2-adrenergic receptor) in the circulating blood cells of four control subjects and four HD-affected patients (Table 3.2). Saturation binding experiments have been performed by using [³H] DPCPX, [³H] MRE 3008F20 and [³H] UK 14304 to label A_1ARs, A_3ARs, and α2-adrenergic receptors in lymphocytes, neutrophils, and platelets, respectively. A single, saturable binding site was detected for all types of receptors examined. Saturation of [³H] DPCPX binding to A_1ARs in lymphocytes showed a K_D value of 1.89 ± 0.11 nM and a Bmax value of 45 ± 3 fmol/mg protein in control subjects, which were very similar to those obtained in HD patients (Table 3.2). Similarly, saturation of [³H] MRE 3008F20 binding to A_3AR adenosine receptors in neutrophils revealed overlapping K_D and Bmax values between controls and HD patients (Table 3.2). Finally, saturation of [³H] UK 14304 binding to α2 adrenergic receptors in the platelets of controls and HD subjects also showed similar K_D and Bmax values. Hence, we concluded that the $A_{2A}AR$ changes detected in HD patients are highly specific for this receptor subtype. In a parallel study performed on a selected number of heterozygous subjects, the coupling of $A_{2A}ARs$ to adenylyl cyclase stimulation and cAMP accumulation in the blood peripheral cells has been determined compared with control subjects. Figure 3.2 shows

typical concentration-response curves to the $A_{2A}AR$ agonist NECA in platelets, lymphocytes, and neutrophils obtained from control and HD heterozygous subjects. A concentration-dependent stimulation of cAMP formation was obtained in the presence of NECA in all cell preparations from control subjects, as shown by EC_{50} values (the agonist concentration eliciting the 50% of maximal cAMP formation) in accordance with literature data (Figure 3.2; Varani et al., 1998). Significantly, NECA EC_{50} values in the peripheral cells from controls (platelets: 308±7 nM, lymphocytes: 199±4 nM, neutrophils: 168±5 nM, n=10) were higher than HD subjects (platelets, 119±13* nM; lymphocytes, 79±10* nM; neutrophils, 85±9* nM; n=10, *P=0.0001), indicating an increased potency of NECA in stimulating cAMP accumulation in HD subjects.

Table 3.1 - Binding parameters of the A$_{2A}$AR in human circulating blood cells of control and HD subjects. *, P < 0.01 vs age-matched controls.

Subjects (males/females)	Age in years	Platelets (P) K_D (nM)	Platelets (P) Bmax (fmol/mg protein)	Lymphocytes (L) K_D (nM)	Lymphocytes (L) Bmax (fmol/mg protein)	Neutrophils (N) K_D (nM)	Neutrophils (N) Bmax (fmol/mg protein)
Controls 44 (21/23)	46.6 ± 1.8	1.04 ± 0.03	115 ± 3	1.08 ± 0.04	67 ± 6	1.14 ± 0.03	86 ± 5
Heterozygous patients 39 (18/21)	45.7 ± 2.1	2.82 ± 0.21*	212 ± 9*	2.92 ± 0.13*	218 ± 5*	3.43 ±1.67*	230 ± 6*
Homozygous patients 3 (2/1)	60.7 ± 5.8	3.12 ± 1.14*	220 ± 11*	3.24 ± 0.24*	219 ± 18*	7.31 ± 0.72*	304 ± 14*
Controls 10 (5/5)	31.1 ± 1.5	1.06 ± 0.05	119 ± 2	1.09 ± 0.06	70 ± 4	1.17 ± 0.02	84 ± 2
Asymptomatic mutation carriers 7 (5/2)	32.9 ± 2.3	3.02 ± 0.53*	211 ± 16*	3.43 ± 0.35*	234 ± 15*	4.14 ± 0.72*	243 ± 10*

Table 3.2 - Binding parameters of the α2-adrenergic receptor, the A₁ARs and A₃ARs in human circulating blood cells from control and HD subjects.

Subjects	Platelets α2 receptors	Lymphocytes A₁ARs	Neutrophils A₃ARs
Controls ***n=4***	$K_D = 3.29 \pm 0.14$ Bmax $= 337 \pm 15$	$K_D = 1.89 \pm 0.11$ Bmax $= 45 \pm 3$	$K_D = 2.24 \pm 0.37$ Bmax $= 451 \pm 20$
HD ***n=4***	$K_D = 3.42 \pm 0.16$ Bmax $= 358 \pm 17$	$K_D = 1.85 \pm 0.11$ Bmax $= 43 \pm 3$	$K_D = 2.07 \pm 0.26$ Bmax $= 461 \pm 27$

Figure 3.1 - Specific binding of [³H]ZM 241385 to platelet, lymphocyte and neutrophil membranes obtained from control (■) and HD heterozygous subjects (●). In inset, the Scatchard plot analysis of specific binding. B and F denote bound radioactivity and free ligand, respectively.
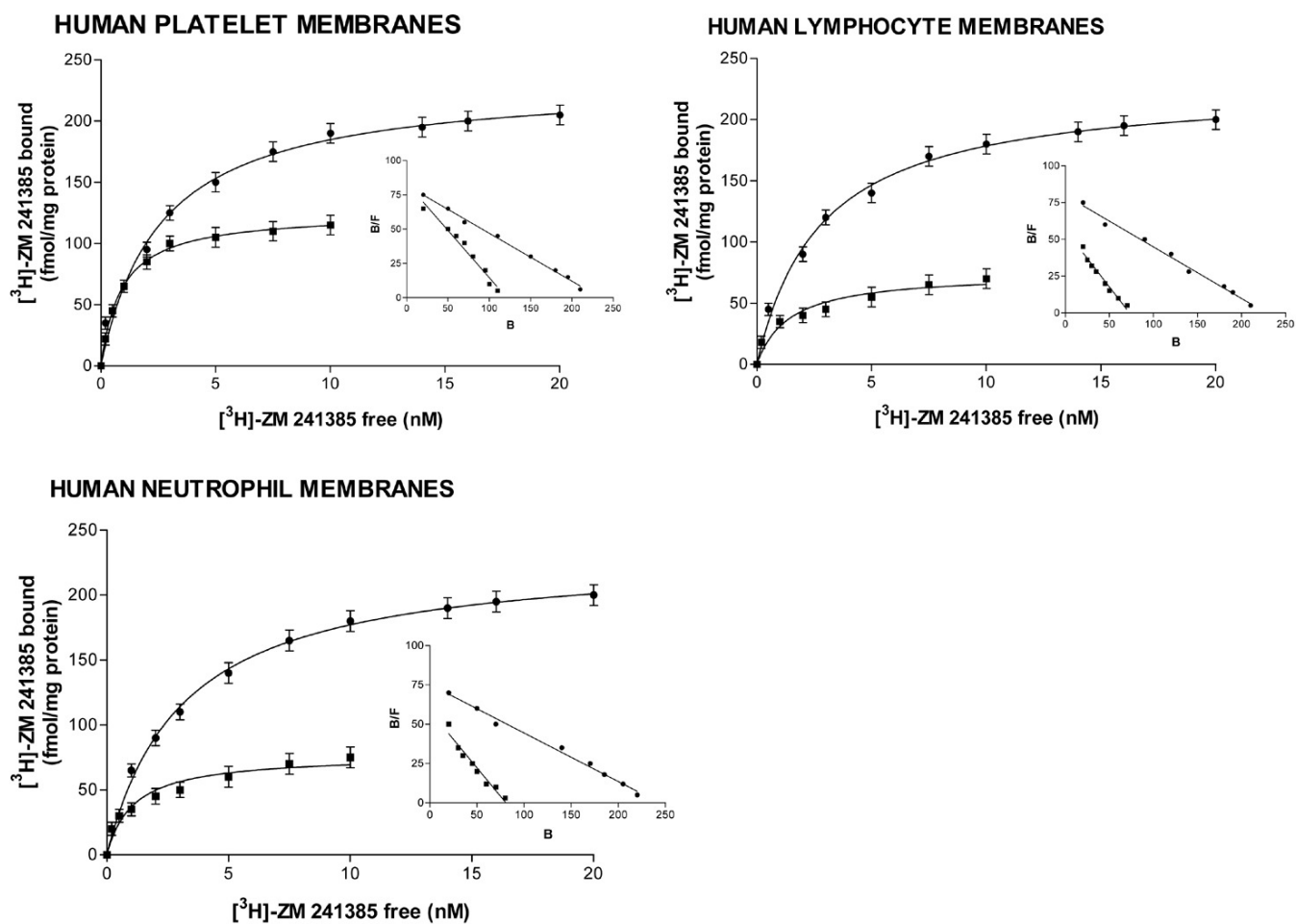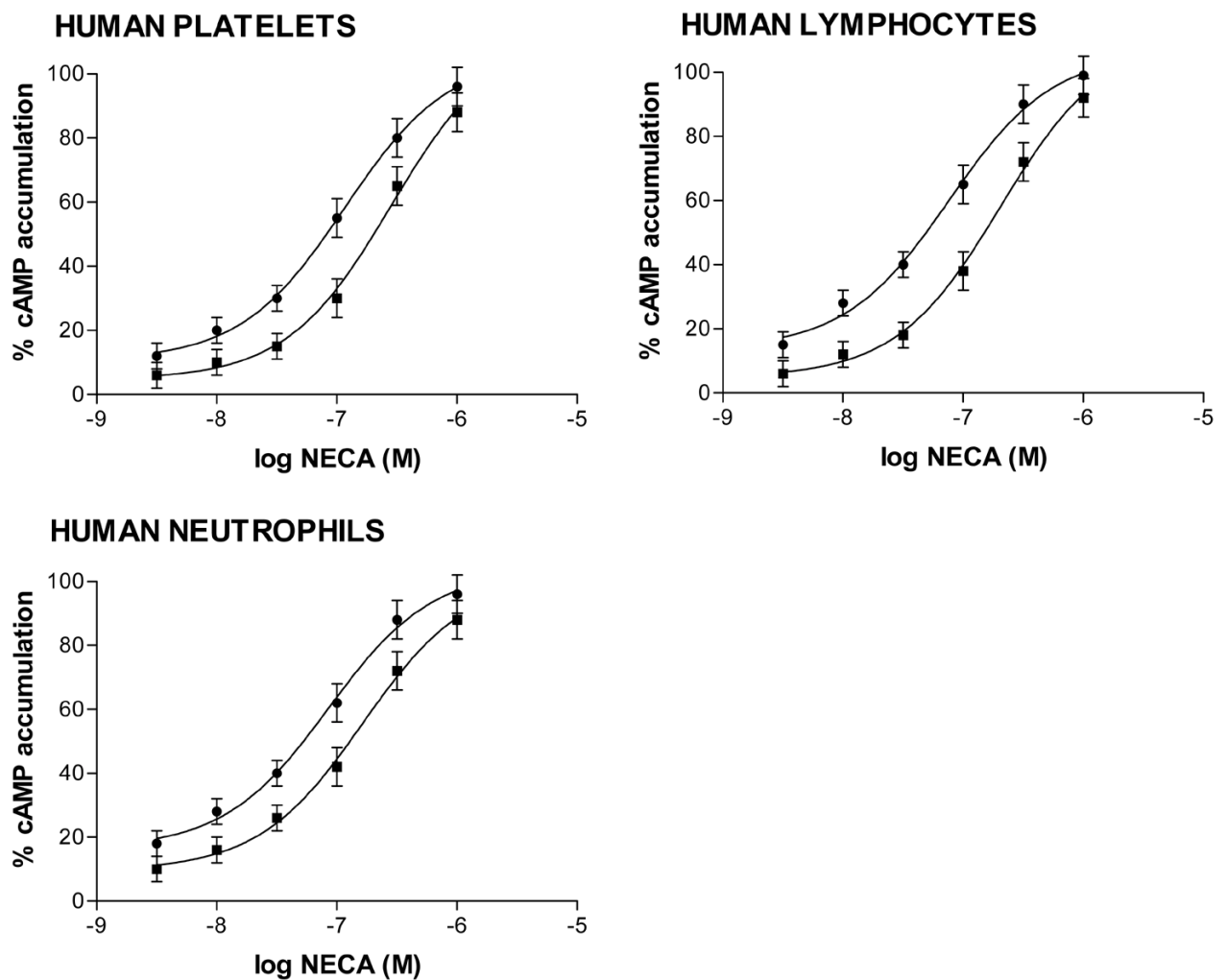
Figure 3.2 - NECA concentration-response curves on cAMP accumulation in platelets, lymphocytes, and neutrophils obtained from control (■) (platelets: $EC_{50} = 308 \pm 7$ nM, lymphocytes: $EC_{50}=199\pm4$ nM, neutrophils: $EC_{50}=168\pm5$ nM, n=10) and HD heterozygous subjects (●) (platelets: $EC_{50}=119\pm13*$ nM, lymphocytes: $EC_{50}=79\pm10*$ nM, neutrophils: $EC_{50}=85\pm9*$ nM, n=10, *P<0.01).

DISCUSSION

In this study we show, for the first time, that peripheral blood cells from HD subjects exhibit an aberrant increase in $A_{2A}AR$ number, as demonstrated by Bmax values significantly higher in these subjects than age-matched healthy controls. This effect was accompanied by an increase of K_D, indicating a decrease in binding affinity. However, the detected increase of K_D is unlike to have any biological significance. In fact, due to the mathematical relationship between Bmax and K_D values and to the method used for the analysis of the results of antagonist ligand binding studies, an increase of receptor density (Bmax) may be accompanied by an increase of the corresponding K_D value (Rovati, 1998). In HD patients, the increase of Bmax was associated with an over-stimulation of $A_{2A}AR$-mediated cAMP production, which is in line with the currently accepted receptor theory. In fact, due to the ratio between extracellular receptors and transductional G-proteins in cellular membranes, an increase of Bmax is expected to result in increased coupling with G-proteins, which, in turn, would result in an increase in function (Kenakin, 1997). No changes of either the A_1ARs or A_3ARs, and of another unrelated GPCR (the α2-adrenergic receptor), have been detected in the circulating cells of HD patients compared with healthy subjects, suggesting that the aberrant $A_{2A}AR$ phenotype in HD is a highly specific receptor change not only within the P1/adenosine receptor family, but also in general within the GPCR superfamily. Globally, these findings recapitulate results obtained in striatal cells expressing human mutant Htt and suggests that the status of the $A_{2A}AR$ in human peripheral cells may reflect a dysfunction present in HD brain. The aberrant $A_{2A}AR$ phenotype was also found in the circulating cells of pre-symptomatic individuals carrying the CAG mutation. These individuals were selected after careful neurological, behavioral, and cognitive assessment and demonstrated to be far away from first HD clinical symptoms. This finding suggests that the aberrant $A_{2A}AR$ behavior may be present at birth or may

accumulate in the pre-symptomatic years. Of note, in spite of the difficulties in directly comparing K_D and Bmax values from HD heterozygotes with those observed in the three homozygous patients available, the latter appeared to express a more severe $A_{2A}AR$ phenotype in neutrophils that reached statistical significance (Table 3.1). As these three homozygotes showed a particularly accelerated disease progression towards disability and no difference in age at onset with respect to the heterozygotes (Squitieri et al., 2003), the biochemical findings herein reported may potentially reflect the clinical and genetic differences between these two groups of patients. A dosage effect of expanded proteins has been demonstrated in other polyglutamine diseases (Gusella and MacDonald, 2000). Further studies are required to evaluate the impact of different disease stages and progression rates on this biochemical parameter and its potential exploitation as a peripheral biomarker of HD. Various reports now indicate that neuronal dysfunction does occur in the pre-symptomatic life stages in HD animal models (Lin et al., 2001) and humans (Lawrence et al., 1998). The findings reported herein that an aberrant $A_{2A}AR$ activity can be measured in the peripheral cells of HD subjects and is already present in pre-symptomatic CAG mutation carriers strengthen the hypothesis that changes of this receptor subtype may occur early in HD pathogenesis and contribute to its progression (Blum et al., 2003). In the brain, $A_{2A}AR$ stimulation of adenylyl cyclase is almost exclusively expressed by striatal medium spiny neurons, where it regulates motor behaviour in cooperation with D_2 dopamine receptors (Ferrè et al., 1993). Consistent with the possible involvement of the $A_{2A}AR$ in HD pathogenesis, selective $A_{2A}AR$ antagonists could reduce the loss of striatal neurons in a pathophysiological animal model of HD and could revert aberrantly increased $A_{2A}AR$ stimulation of adenylyl cyclase in mutant-Htt expressing striatal cells. This evidence highlights the $A_{2A}AR$ as an interesting target for HD therapy. Although further studies are needed to evaluate the actual impact of selective $A_{2A}AR$ antagonists on the

survival of striatal neurons, we speculate that an early treatment with such compounds may be able to reduce or retard HD-associated neuronal loss. Although this study demonstrates that an aberrant $A_{2A}AR$ behavior is present in peripheral, circulating cells of HD subjects, it remains to be established whether this measurable receptor activity can be taken as a marker of disease severity and/or progression. For other CNS disorders, alterations of proteins specifically involved in the disease, such as dopamine receptors in migraine (Barbanti et al., 2000) and schizophrenia (Ilani et al., 2001) and amyloid precursor protein (APP) in Alzheimer's disease (Di Luca et al., 1998), have been found to be reproduced in the peripheral blood cells of patients.

CHAPTER 4:

Biological abnormalities of peripheral A_{2A}ARs in a large representation of polyglutamine disorders and Huntington's disease stages.

INTRODUCTION

HD is a dominantly inherited neurodegenerative disease featuring progressive worsening motor dysfunctions, psychiatric disturbances and cognitive impairment due to brain cell loss. Although the GABAergic enkephalin neurons of the basal ganglia and neurons in the cerebral cortex are primarily affected, a number of reports now indicate that mutant huntingtin, which is ubiquitously expressed, evokes profound molecular alterations in a variety of extraneural tissues. This evidence has led to the intense search in patients of peripheral changes that may be directly linked to the clinical manifestations and outcome of the disease, as to improve early detection of disease progression and efficacy of future therapeutic interventions. The ultimate goal will be the availability of diagnostic biomarkers that reflect pathophysiological mechanisms to be targeted in peripheral cells and that are linked to disease status.

Toward this aim we reported the first evidence of a peripheral dysfunction in human HD subjects consisting of an aberrant $A_{2A}AR$ receptor function in freshly isolated peripheral blood cells. The $A_{2A}AR$ is a membrane receptor with a main locus on the GABAergic-striatal neurons which degenerate in HD. Its role is related to the control of movement and in particular to the inhibition of involuntary movements. We originally reported that an aberrant amplification of $A_{2A}AR$-stimulated adenylyl cyclase is present in a cellular model of HD while data in HD mice showed an increase of $A_{2A}AR$ receptor density (Bmax) and $A_{2A}AR$-stimulated adenylyl cyclase activity in the striatum.

On these grounds we assessed whether the aberrant peripheral $A_{2A}AR$ phenotype could be detected also in human peripheral cells and may possibly represent a biomarker for the disease. We reported that, likewise in the mice, the $A_{2A}AR$ density was substantially increased in all tested cells (lymphocytes, neutrophils and platelets) from 51 HD patients and seven subjects at pre-symptomatic stage versus 58 controls that remained remarkably similar in their $A_{2A}AR$ receptor Bmax value.

Here we aimed at extending the analyses of $A_{2A}AR$ receptor activity to include a more detailed transversal study where different disease stages were represented, as well as at testing HD patients and larger number of pre-symptomatic subjects enrolled from different clinical sites. In addition, we tested whether the $A_{2A}AR$ dysfunction was present in other inherited neurological diseases, such as other polyQ- (Spinocerebellar Ataxia, SCA, type 1 and 2) or not polyQ-linked (Freidreich Ataxia, FRDA) neurodegenerative disorders. Our assumption was that diseases with a common pathogenic mechanism (for example an expanded CAG repeat) could exhibit the same dysfunction, a finding that may have implications in the development of future therapeutic interventions. Though it is often unpredictable whether or not a peripheral abnormality could be of clinical utility, the identification in peripheral cells of a measurable significant change in a biological process might arguably serve as a useful endpoint to measure the efficacy of therapeutics targeting pathological mechanisms initiated by the mutation.

MATHERIALS AND METHODS

Patients

Individuals were recruited for study and clinically assessed at the Henri Mondor Hospital, Créteil (France), at the National Neurological Institute Carlo Besta, Milan (Italy), at the Ferrara University Hospital Blood Bank (Italy) and at the University Hospital of Wales (UK). During the visit, 30 ml of blood was withdrawn. All eligible participants received verbal and written information about the study and signed an informed consent form. For symptomatic HD patients with severe cognitive impairment, the informed consent was obtained from their relatives. The study protocol was approved by the Ethics Committee of each participating centre.

A total of 252 individuals were recruited in the study period. Of these, 94 were HD patients (73 from Henri Mondor Hospital, 21 from Neurological Institute Besta); 32 were pre-HD (4 from Henri Mondor Hospital, 17 from Neurological Institute Besta, 11 from University of Wales); 12 were SCA1, 27 SCA2, 24 FRDA (all from Neurological Institute Besta); and 63 control individuals (58 from Ferrara University Hospital Blood Bank University of Ferrara and 5 from Neurological Institute Besta). Clinical and genetic characteristics of participants are illustrated in Table 4.1. Patients from the different disease groups were representative of the disease spectrum in terms of gender, disease duration and clinical compromise. As expected, pre-HD had a median age lower than HD. Also FRDA age was younger, as expected in an infantile-juvenile disease. Due to the similar characteristics and limited numbers, SCA1 and SCA2 patients have been combined in subsequent analyses. Disease-specific inclusion criteria were as follows:

(a) Symptomatic HD patients (HD). Patients with manifest clinical signs and symptoms of the disease, with UHDRS motor score ≥ 5 and positive at molecular test for the presence of a CAG triplet repeat number ≥ 35 in the Huntington gene.

(b) Pre-symptomatic HD individuals (Pre-HD). These individuals were selected among those who decided to undergo the program for pre-symptomatic genetic testing according to the International Huntington's Association (IHA) and World Federation of Neurology (WFN) guidelines (IHA and WFN Research Group for HD, 1994). People with more that 35 CAG triplet repeats, no clinical signs/symptoms of the disease, UHDRS motor score < 5 were recruited for the study.

(c) Patients with spinocerebellar ataxia type 1 (SCA1) and type 2 (SCA2). Patients with clinical signs of cerebellar ataxia, ICARS score ≥ 2 and positive molecular test, showing CAG triplet repeats ≥ 38 in SCA1 gene, or ≥ 32 in SCA2 gene.

(d) Patients with Friedreich ataxia (FRDA). Patients with clinical signs of cerebellar ataxia (ICARS score ≥ 2) and with GAA triplet repeat ≥ 66 on both FRDA gene alleles.

(e) Control individuals. Volunteers with no neurodegenerative disorders were enrolled from Ferrara University Hospital Blood Bank and Neurological Institute Besta.

Patients and controls aged less than 18 years or/and having known major medical conditions, in addition to the primary genetic disorder, were excluded.

All the patients were evaluated by a standard neurological examination. Detailed family history, age of onset of symptoms, medications and other relevant clinical information were collected. Clinical status was determined using the Unified Huntington Disease Rating Scale (UHDRS) in HD patients (Marder et al., 2000) and the International Cooperative Ataxia Rating Scale (ICARS) in SCA1, SCA2 and FRDA patients (Trouillas et al., 1997).

Genetic assessment

DNA for genetic diagnosis was extracted from venous lymphocytes. Genetic tests for HD, SCA1, SCA2 and FRDA were performed in the laboratory of molecular genetic of the division of Biochemistry and Genetics of the Neurological Institute Carlo Besta according to the published methods to study trinucleotide repeat diseases (Gellera et al., 1996). HD tests were carried out in the laboratory of Medical Genetics, University Hospital of Wales and in different approved centers for genetic testing in HD included in the Huntington French Speaking Group for the Henri Mondor Hospital subjects (Créteil).

$A_{2A}AR$ receptor study: preparation of peripheral blood cells or membrane suspensions

Peripheral venous blood samples were obtained from patients and healthy controls. The isolation of the various cell fractions (platelets, lymphocytes, neutrophils) started no later than 6–8 hours after the samples had been taken. Membranes from human platelets, lymphocytes, and neutrophils were prepared as previously described (Varani et al., 1997;1998) and used for radioligand binding assays. Washed platelets, lymphocytes, and neutrophils were prepared to evaluate cAMP levels (Varani et al., 1997;1998).

[^{3}H]-ZM 241385 binding assay to A$_{2A}$ARs

A$_{2A}$AR parameters were evaluated by means of receptor binding with A$_{2A}$AR radioligand antagonist [^{3}H] ZM 241385 (Tocris Cookson Ltd., Bristol, UK). The binding parameters of the human A$_{2A}$ARs were determined in lymphocytes by a standard procedure (Varani et al., 1998).

Briefly, membranes were incubated with 8–10 different concentrations of the A$_{2A}$AR antagonist radioligand [^{3}H] ZM 241385 for 60 min at 4 °C. Non-specific binding was determined in the presence of NECA 10 μM. Bound and free radioactivity were separated by filtering the assay mixture on Whatman GF/B glass fiber filters with a Micro Mate 196 Cell harvester (Packard Instrument Co.). The filter bound radioactivity was measured using a microplate scintillation counter (Top Count, Meriden, CT) at an efficiency of 57% with Micro Scint 20. Binding parameters such as affinity (K$_D$) and receptor density (Bmax) were calculated by computer-assisted analysis.

Measurement of cyclic AMP levels in peripheral blood cells

Adenylyl cyclase activity was evaluated by incubating washed peripheral blood cells (either platelets or lymphocytes or neutrophils) in the absence or presence of 6–8 different concentrations of the A$_{2A}$AR agonist NECA. Cells were suspended in 0.5 ml

of buffer containing 2 I.U. of adenosine deaminase and 0.5 mM 4-3-butoxy-4-methoxybenzyl-2-imidazolidinone (Ro 20-1724) as a phosphodiesterase inhibitor and were preincubated in a shaking bath at 37°C. NECA was added to the mixture and the incubation continued for another 10 min. The reaction was terminated by addition of cold 6% trichloroacetic acid (TCA). The TCA suspension was centrifuged at 2000 x g for 10 min at 4°C, and the supernatant was extracted four times with water-saturated diethyl ether. The final aqueous solution was tested for cAMP levels by a competition protein-binding assay. Samples of cAMP standards (0–10 pmol) were added to each test tube containing the binding protein (previously prepared from beef adrenals and incubated at 4°C for 150 min). After addition of charcoal, the samples were centrifuged at 2000 x g for 10 min. The clear supernatant was mixed with Atomlight (Perkin-Elmer, Beltsville, MD) and counted by using a Beckman liquid scintillation counter.

Statistical analysis

A weighted nonlinear least-squares curve-fitting program LIGAND (Munson and Rodbard, 1980) was used for computer analysis of the data from the binding saturation experiments, as previously described. EC_{50} values in the cAMP assay were calculated with the non-linear least-squares curve-fitting program Prism (Graph PAD, San Diego, CA). Nonparametric tests (Mann Whitney U) were used to compare each class of patients and healthy controls. Differences were considered significant at a value of $P < 0.01$. All data are reported as mean ± SEM.

RESULTS

Binding characterization of $A_{2A}ARs$

Figure 4.1 shows [^{3}H]-ZM 241385 saturation curves in membranes prepared from platelets from HD, pre-HD, SCA (1 and 2) and FRDA compared with control subjects.

Figures 4.2 and 4.3 show [^{3}H]-ZM 241385 saturation curves in membranes of lymphocytes and neutrophils of the same subjects, respectively. In all the blood cells studied, A$_{2A}$AR binding was saturable and indicated the presence of a single receptor population characterized by affinity and density values that are in agreement with those previously reported in the literature for these human cell populations (Table 4.2; Varani et al., 2003). In particular, as expected, this analysis in control healthy subjects revealed a uniform level of A$_{2A}$ARs in the peripheral blood cells. Analysis of the same parameters in HD subjects revealed a statistically significant increase in A$_{2A}$ARs density (Bmax) in all cell populations when compared to controls ($P<0.01$ versus control, Table 4.1). The increase in Bmax was accompanied by an increase in K$_D$ values ($P<0.01$ vs. control, Table 4.2).

Interestingly, analysis of A$_{2A}$AR K$_D$ and Bmax values in pre-symptomatic HD mutation carrier revealed highly increased A$_{2A}$AR parameters in all three blood cell types when compared to controls (Table 4.2). In addition, also in the membranes obtained from the patients affected by SCA1 and SCA2, Bmax values for the A$_{2A}$ARs were significantly higehr than in control subjects and strictly similar to those obtained from HD patients.

On the contrary, analysis of the A$_{2A}$AR parameters in the blood cells of patients affected by FRDA revealed K$_D$ and Bmax values similar to those obtained from control subjects and consequently different from HD and SCA subjects (Table 4.2).

cAMP assays in peripheral blood cells

In platelet, lymphocytes and neutrophils obtained from HD, pre HD, SCA (1 and 2) and FRDA patients, the coupling of A$_{2A}$ARs to adenylyl cyclase stimulation and cAMP accumulation has been determined compared with control subjects. Figure 4.4 shows typical concentration-response curves of the A$_{2A}$AR agonist NECA in platelets, lymphocytes, and neutrophils obtained from examined controls and patients. A

concentration-dependent stimulation of cAMP formation was obtained in the presence of NECA in all cell preparations from control subjects, as shown by EC_{50} values (the agonist concentration eliciting the 50% of maximal cAMP formation) in accordance with literature data (Table 4.3; Varani et al., 1998).

Interestingly, NECA EC_{50} values in the peripheral cells from controls were higher than all HD subjects (symptomatic or pre- symptomatic), indicating an increased potency of NECA in stimulating cAMP accumulation in HD subjects (Table 4.3). Interestingly, the potency of NECA was increased also in the peripheral blood cells of SCA subject when compared to controls. On the contrary, in platelets, lymphocytes and neutrophils from FRDA subjects, the potency of NECA was strictly similar to that obtaine in control healthy subjects.

Effect of pharmacological treatments

Many of the patients examined in this study were treated with one or more drugs which included selective serotoninergic reuptake inhibitors (SSRI), tricyclic compounds, neuroleptic, benzodiazepine and antiepileptics for the neurological phenotype and antioxidant, anti-hypertensive and other non-specific drugs as anti-inflammatory drugs or hormones (Table 4.4). The comparison of the $A_{2A}AR$ binding parameter in differently treated patients revealed that the status of $A_{2A}AR$ is not affected by different pharmacological treatments.

Table 4.1 – Clinical and genetic characteristics of patients and healthy controls.

Characteristic	HD (n=94)	Pre-HD (n=32)	SCA 1 (n=12)	SCA 2 (n=27)	FRDA (n=24)	Controls (n=63)
Male/Female	50/44	16/16	5/7	13/14	12/12	29/34
Age (yrs)	49 (25–76)	36 (22–55)	44 (29–63)	41 (24–66)	30 (18–55)	45 (24–64)
Disease duration (yrs)	5 (1–22)	0	8 (3–15)	7 (0–25)	19 (6–41)	--
CAG/GAA[a] repeats normal allele	17 (10-33)	18 (10-21)	29 (22-33)	22 (22-28)	605 (330-930)[a]	--
CAG/GAA[a] repeats expanded	44 (39-54)	43 (38-52)	48 (38-62)	40 (35-48)	803 (560-1200)[a]	--
Modified Rankin	2 (0-4)	--	3 (1-5)	1 (1-2)	4 (2-4)	--
UHDRS Motor Score	33 (5-96)	0 (0-4)	--	--	--	--
TFC	11 (0-13)	13 (12-13)	--	--	--	--
ICARS	--	--	36 (8-84)	27 (2-73)	65 (22-94)	--

Data are expressed as median (min-max)

Table 4.2 - **Binding parameters of A_{2A}ARs in platelets, lymphocytes and neutrophils of examined subjects. *, P < 0.01 vs control subjects.**

	Platelets		Lymphocytes		Neutrophils	
	K_D (nM)	Bmax (fmol/mg protein)	K_D (nM)	Bmax (fmol/mg protein)	K_D (nM)	Bmax (fmol/mg protein)
Controls (n=63)	1.15 ± 0.10	62 ± 6	1.10 ± 0.10	65 ± 6	1.40 ± 0.15	89 ± 8
HD (n=94)	2.21 ± 0.20*	173 ± 18*	2.85 ± 0.29*	253 ± 22*	3.10 ± 0.30*	267 ± 25*
Pre-HD (n=32)	2.32 ± 0.24*	178 ± 16*	2.78 ± 0.25*	201 ± 16*	3.04 ± 0.30*	240 ± 20*
SCA (n=39)	2.40 ± 0.22*	187 ± 20*	2.92 ± 0.30*	236 ± 20*	2.98 ± 0.30*	249 ± 23*
FRDA (n=24)	1.05 ± 0.10	66 ± 5	1.20 ± 0.11	62 ± 6	1.20 ± 0.10	82 ± 8

Table 4.3 - EC_{50} values of NECA on cAMP stimulation in platelets, lymphocytes and neutrophils of examined subjects. *, $P < 0.01$ vs control subjects.

	Platelets (EC_{50}, nM)	Lymphocytes (EC_{50}, nM)	Neutrophils (EC_{50}, nM)
Controls (n=63)	300 ± 28	205 ± 20	246 ± 22
HD (n=94)	80 ± 8*	60 ± 6*	70 ± 7*
Pre-HD (n=32)	84 ± 7*	65 ± 6*	78 ± 8*
SCA (n=39)	76 ± 7*	55 ± 6*	68 ± 7*
FRDA (n=24)	320 ± 30	210 ± 0.22	230 ± 25

Table 4.4 - Pharmacological therapies of patients involved in the study.

	No drugs	SSRI	Tryciclic	Neuroleptic	BZD	Antiepyleptic	Antioxidant	Hypertensive	Antiarythmic	Other
HD (94)	11	29	25	29	34	4	3	3	17	27
Pre-HD (32)	25	1			1					5
SCA (39)	17	7	1	4	5	4	8	2	2	16
FRDA (24)	2	3			5	1	16	1	4	4

Figure 4.1 - Saturation curves (A) and Scatchard Plot (B) of [^{3}H]-ZM241385 in platelets from the examined subjects.
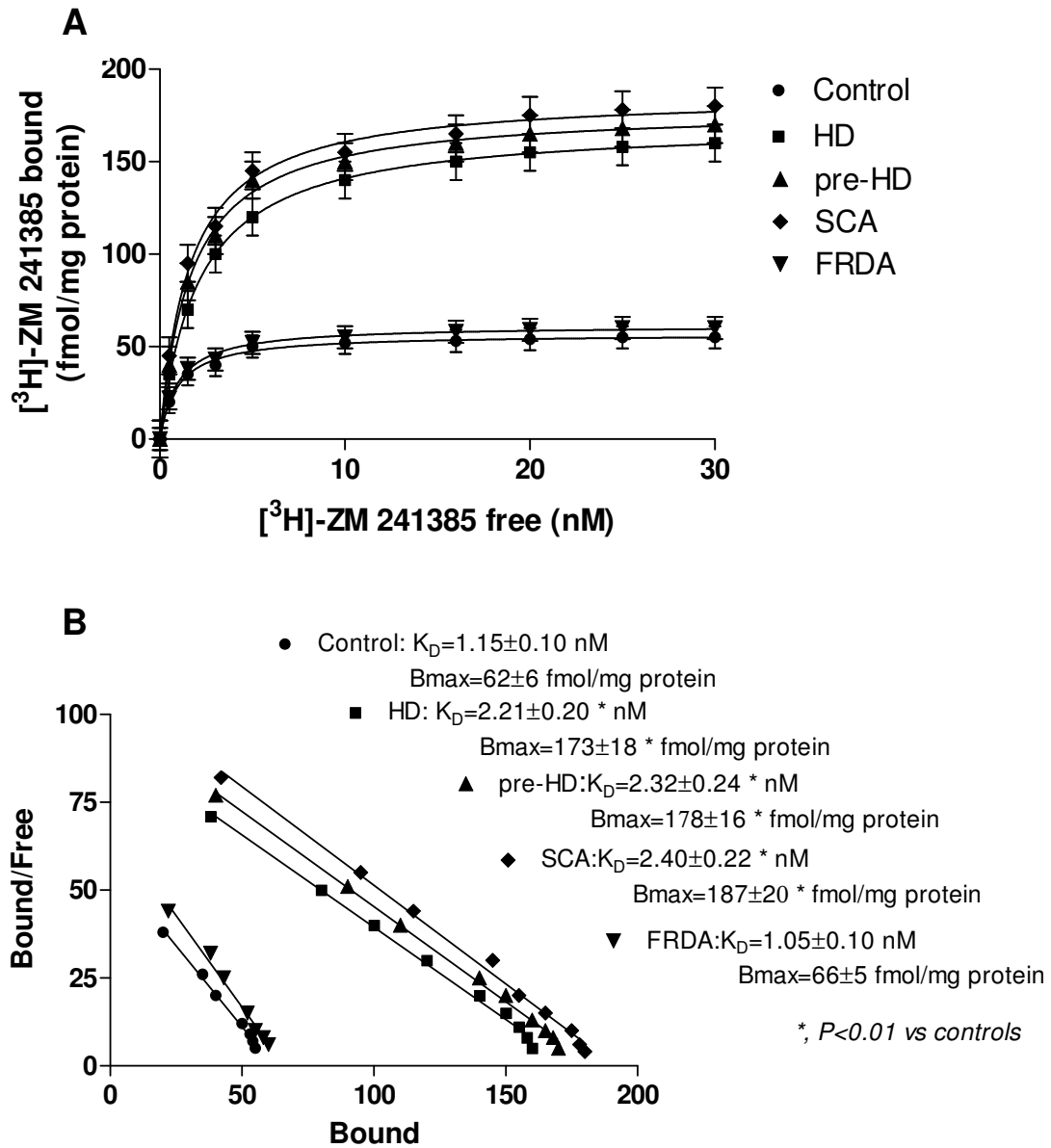
Figure 4.2 – Saturation curves (A) and Scatchard Plot (B) of [^{3}H] - ZM241385 in lymphocytes from the examined subjects.
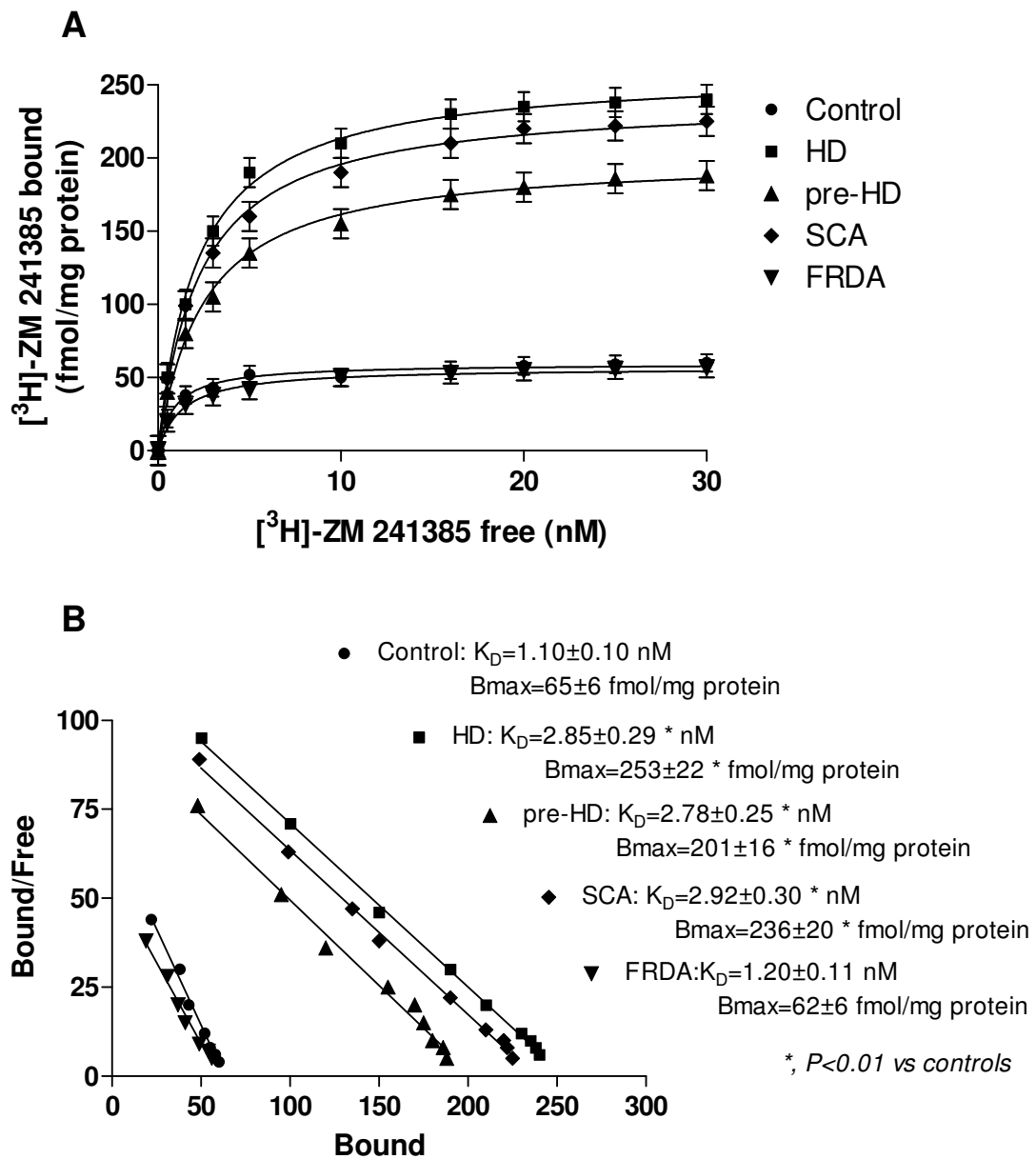
Figure 4.3 - Saturation curves (A) and Scatchard Plot (B) of [³H] - ZM241385 in neutrophils from the examined subjects.
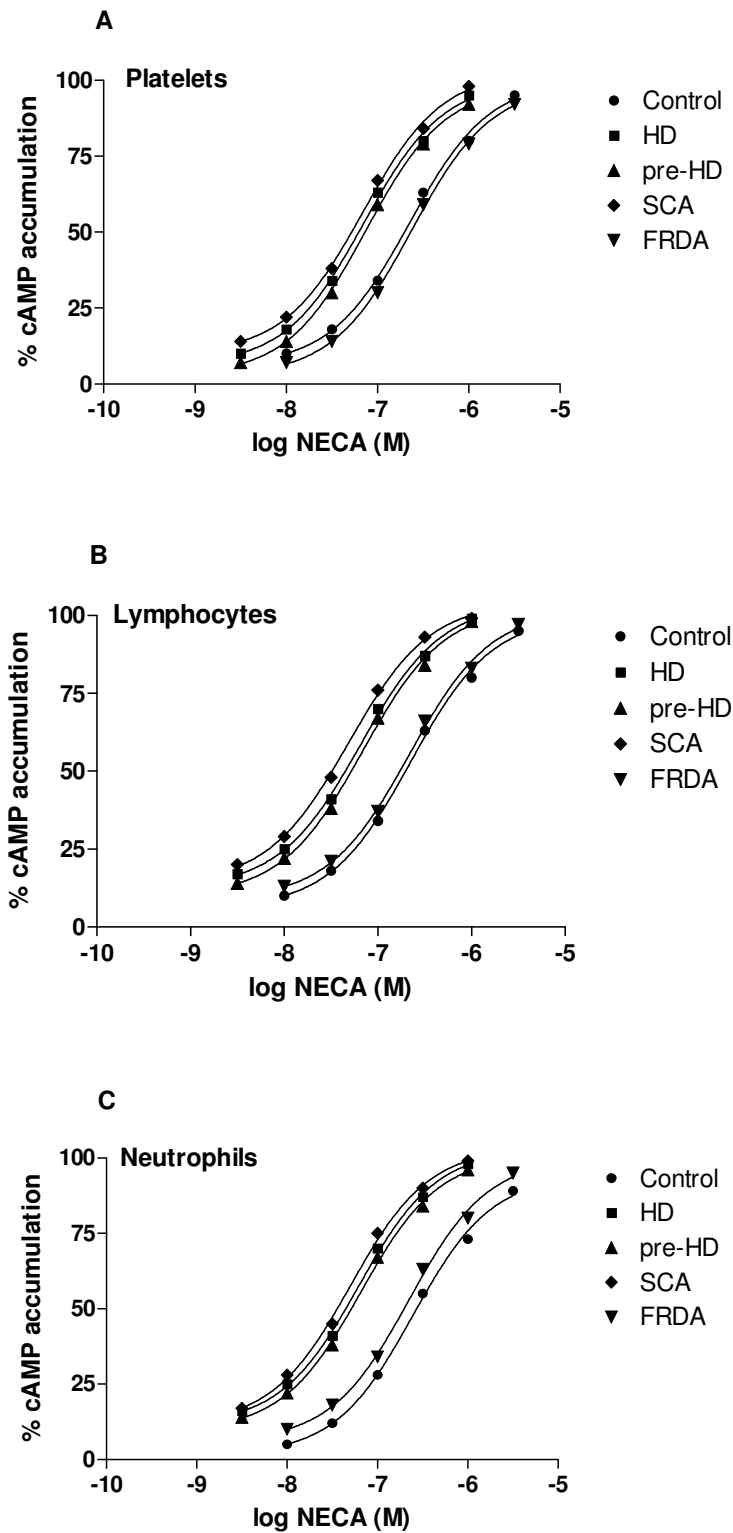
A

B

Control: K_D=1.40±0.15 nM
Bmax=89±8 fmol/mg protein

HD: K_D=3.10±0.30 * nM
Bmax=267±25 * fmol/mg protein

pre-HD: K_D=3.04±0.30 * nM
Bmax=240±20 * fmol/mg protein

SCA: K_D=2.98±0.30 * nM
Bmax=249±23 * fmol/mg protein

FRDA: K_D=1.20±0.10 nM
Bmax=82±8 fmol/mg protein

*, P<0.01 vs control
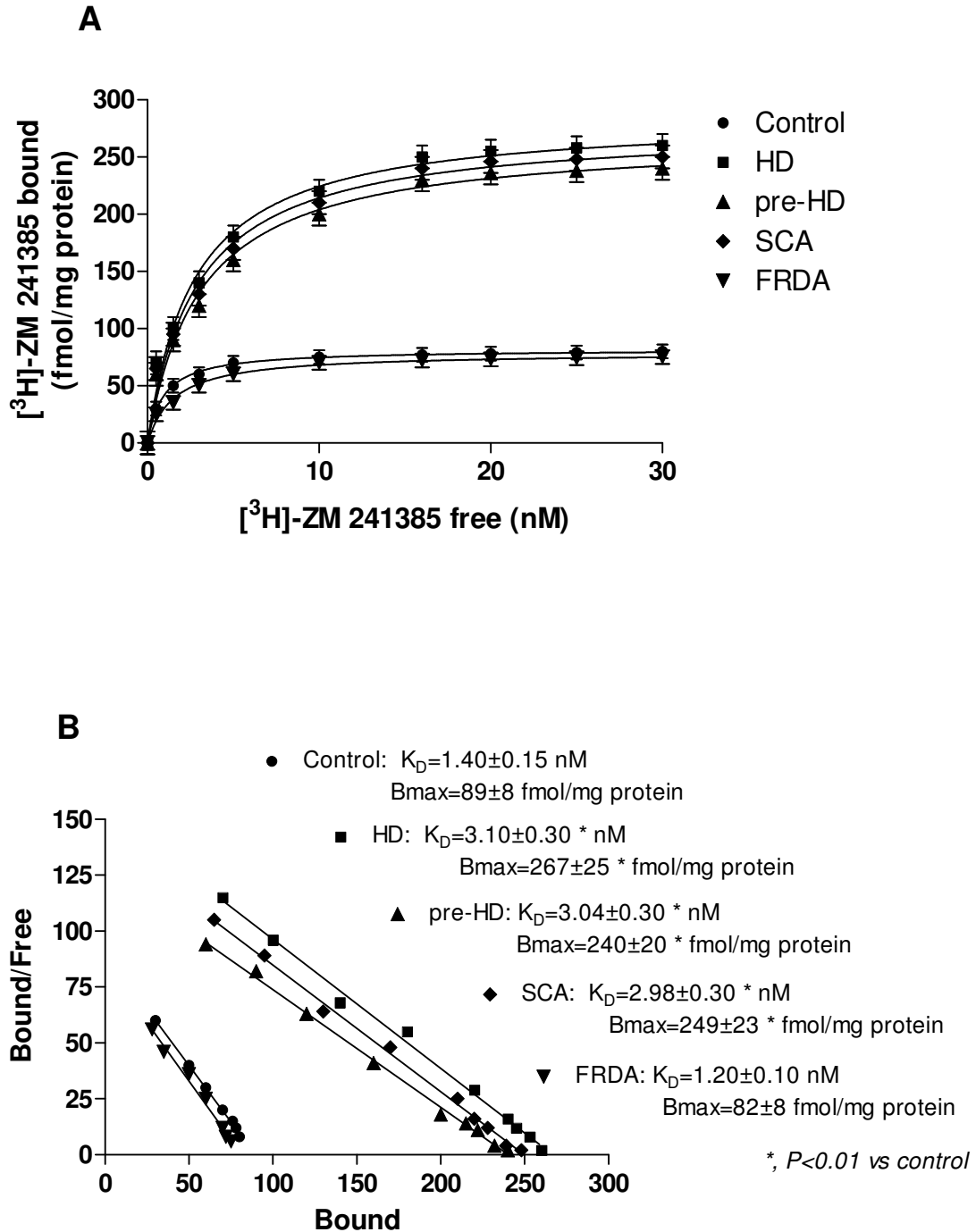
Figure 4.4 – Dose response curves of NECA in plateles (A), lymphocytes (B) and neutrophils (C) from the examined subjects.

DISCUSSION

In neurology, the search for objectively measurable tests of disease progression is playing an increasing role. In Huntington's disease, the evidence that the mutant protein is widely distributed and present in peripheral cells has fueled a large number of attempts aimed at identifying disease-specific changes in biological processes that could be detected in easily accessible cells. The hope is that these could then be included in therapeutic trials as molecular markers of the action of the mutant protein and, as a consequence exploited to evaluate the effectiveness of novel potential therapeutic agents. In addition, a change in a peripheral parameter that is embedded into a pathophysiological mechanism may be indicative of a pathway of clinical and biological relevance and possibly represent itself a target of therapeutic interest.

So far, there are limited reports of peripheral changes in human HD samples and far less that correlate with disease status. The most promising results likely derive from a non-hypothesis-driven study of global changes in gene expression in blood samples from 17 HD subjects (late pre-symptomatic and symptomatic) and 14 gender-matched controls (Borovecki et al., 2005). Among the critical outcomes of this study is the evidence that a set of 12 genes distinguishes normal controls from HD subjects and that early pre-symptomatic HD subjects disclose an expression pattern similar to controls, whereas the late pre-symptomatic subjects had patterns similar to symptomatic HD patients (Borovecki et al., 2005). These findings suggested that a subset of mRNAs isolated from whole blood could help in monitoring clinical onset of this neurodegenerative disease.

We have previously assessed that an alteration in $A_{2A}AR$ binding activity is present in peripheral blood cells from HD patients and a small number of pre-HD, but that study was not addressing the issue of whether the detected $A_{2A}AR$ alteration is specific to HD, and possibly correlates to disease stage and/or to other HD clinical and genetic

parameters. The results obtained in this new study show three new findings: (i) all HD patients who tested positively for the polyQ expansion also presented an altered Bmax value with respect to control subjects with no significant differences in Bmax across patients with different disease stages; (ii) the 32 HD-positive pre-manifesting subjects did not differ from symptomatic HD patients with respect to Bmax values; (iii) patients with other polyQ diseases as SCA1 and SCA2 showed the very same change in $A_{2A}AR$ Bmax values as HD patients and differed from controls and patients with other neurodegenerative disorders such as Friedreich's ataxia.

The fact that the Bmax values are similarly altered in HD patients at different stages and in pre-HD subjects indicates that this change is directly related to the immediate cellular effects of the mutation and not to the pathological state of the patients or disease progression. The presence of the very same change in the $A_{2A}AR$ in SCA1 and SCA2 patients reinforces the assumption that our findings substantially represent a cellular toxic effect of the polyQ proteins.

Similar $A_{2A}AR$ alteration was found evaluating the functionality of the receptor through cAMP accumulation assay. Indeed, increased potency of NECA was found in peripheral blood cells of HD, pre-HD and SCA subject, but not in FRDA subjects, when compared to control healthy subjects

We can conclude that the observed alteration of $A_{2A}AR$ in platelets, lymphocytes and neutrophils links with the presence of the poliglutamine proteins and may be of potential interest in the search of novel drugs aimed to counteract the toxic effect of the mutation.

GENERAL CONCLUSIONS

Huntington's disease (HD) is a devastating autosomal dominant neurodegenerative disease caused by a CAG trinucleotide repeat expansion encoding an abnormally long polyglutamine tract in the huntingtin protein. Expression of mutant huntingtin leads to the selective death of the medium spiny neurons in the neostriatum, resulting in the appearance of generalized involuntary movements, the main phenotypic alteration of HD. A_{2A}ARs are largely expressed in these neurons and modulate different processes in the cental nervous system, suggesting a possible involvement of these receptors in Huntington's disease.

In the first part of this study we have investigated the presence and functionality of A_{2A}ARs in striatal cells expressing different form of mutant huntingtin. We found an aberrant amplification of A_{2A}ARs-stimulated adenylyl cyclase specifically associated with the expression of mutant huntingtin. Despite no changes of Bmax values, binding studies revealed a moderate but statistically significant increase of ligand binding affinity in cells expressing mutant huntingtin, which suggests that mutant, but not wild-type huntingtin, may interfere with the kinetics of A_{2A}AR binding by endogenous adenosine and may influence the conformation of the ligand recognition site. From our data it is also clear that changes of A_{2A}AR signaling are much more evident in cells expressing truncated than full-length mutant huntingtin. This finding may have intriguing functional implications, because the N548 fragment expressed in these cells mimics one of the potential fragments suggested to be produced by proteolytic cleavage of huntingtin and which may be required for the expression of mutant huntingtin cytotoxicity and aggregate formation.

In the second part of the study we investigated if the aberrant phenotype of $A_{2A}AR$ was also present in R6/2 mice. These animals express exon 1 of the human Huntingtin gene with an expanded CAG repeat length of 141–157 under the influence of the human promoter. The R6/2 model has many of the temporal, behavioral and neuropathological features observed in HD patients, such as jerky movements and striatal atrophy. Analysis of striatal $A_{2A}AR$ binding and adenylyl cyclase activity in R6/2 mice of different developmental ages in comparison with age-matched wild-type animals showed a transient increase in $A_{2A}AR$ density (Bmax) and $A_{2A}AR$ -dependent cAMP production at early presymptomatic ages (7–14 postnatal days, PND) that is, well before the onset of motor symptoms. Despite the increased receptor density observed in radioligand binding studies in R6/2 versus wild-type mice (PND 9–14), we did not find any significant change of $A_{2A}AR$ mRNA by employing real time PCR. This suggests that, at this developmental age, receptor turnover rather than receptor synthesis may be altered in these animals.

In the subsequent study we have shown, for the first time, that peripheral blood cells from HD subjects exhibit an aberrant increase in $A_{2A}AR$ number, as demonstrated by Bmax values significantly higher than age-matched healthy controls. In platelets, lymphocytes and neutrophils of HD patients, the increase of Bmax was associated with an over-stimulation of $A_{2A}AR$-mediated cAMP production. The aberrant $A_{2A}AR$ phenotype was also found in the circulating cells of pre-symptomatic individuals carrying the CAG mutation. This opened the possibility that $A_{2A}AR$ may represent a novel potential biomarker of HD, useful for monitoring disease progression and assessing the efficacy of novel neuroprotective approaches.

The last part of the study was performed in a larger number of HD patients at different stages of the disease, in pre-symptomatic carrier of the mutation, in patients affected by other polyglutamine disease such as Spinocerebellar Ataxia 1 (SCA1), Spinocerebellar

Ataxia 2 (SCA2) and in patients affected by a non-polyglutamine disease, i.e. Friedreich ataxia.

We found that all HD patients who tested positively for the polyglutamine expansion also presented an altered Bmax value when compared to control subjects with no significant differences in Bmax across patients with different disease stages. Furthermore, $A_{2A}AR$ density of pre-symptomatic carrier of the mutation did not differ from symptomatic HD patients. In addition, patients with other polyglutamine diseases such as SCA1 and SCA2 showed a strictly similar change in $A_{2A}AR$ Bmax values as HD patients and differed from controls and patients with other neurodegenerative disorders such as Friedreich's ataxia.

Overall these data demonstrate that an aberrant $A_{2A}AR$ phenotype is present in Huntington's disease and this seems to be related with the presence of the expanded GCA triplet. The amplification of the signal transduction system of $A_{2A}ARs$ suggests that the use of selective $A_{2A}AR$ antagonists could be beneficial in the treatment of Huntington's disease as well as in other related polyglutamine diseases. In addition, the alteration of $A_{2A}ARs$ in peripheral blood cells of patient with polyglutamine diseases suggests that this receptor could be an easily accessible biomarker for the evaluation of the efficacy of potential new therapies.

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