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MOLECULAR APPROACH TO NEURODEGENERATIVE DISORDERS: ROLE OF NOCICEPTIN/ORPHANIN FQ - NOP SYSTEM IN PARKINSON AND EPIGENETIC MECHANISMS IN ALZHEIMER'S DISEASE

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ABSTRACT

With life expectancies increasing around the world, populations are getting age and neurodegenerative diseases have become a global issue. Neurodegenerative disorders are the result of abnormalities in the transport, degradation and aggregation of particular types of proteins in the brain. There are currently no known biomarkers or preventative strategies for neurodegenerative diseases. This provides much opportunity for ground-breaking research. For this reason we have focused our attention on the two most important neurodegenerative diseases: Parkinson's and Alzheimer's.

Parkinson's disease (PD) is a chronic progressive neurodegenerative movement disorder of multi-factorial origin. Environmental toxins as well as agricultural chemicals like Rotenone, Paraquat (PQ) and Maneb (MB) have been associated with PD. Has been observed that N/OFQ contributes to both neurotoxicity and symptoms associated with PD and that pronociceptin (ppN/OFQ) gene expression is up-regulated in rat SN of 6-OHDA and MPP induced experimental parkinsonism. First, we investigated the role of N/OFQ-NOP system in the pathogenesis of PD in an animal model developed using PQ and/or MB. Adult male Sprague–Dawley rats were injected intraperitoneally (i.p.) twice a week for 4 weeks with: saline solution, PQ + MB HD (10 + 30 mg/kg) and PQ + MB LD (5 + 15 mg/kg). Rat weight, locomotion activity and specific behaviours were scored and evaluated. We determined tyrosine hydroxylase (TH), alpha synuclein (SNCA), P-ERK ½, CREB, GAD 65/67 levels in the substantia nigra (SN) and caudate putamen (CP) by Western Blotting. Quantification of N/OFQ-NOP and PDYN/KOP system, as well parkin gene expression in SN and CP was performed by Real Time RT-PCR. Weight and locomotion activity decreased significantly in all treated animals compared with controls, and animal behavior showed a significant difference between both PQ + MB groups versus control group. A significantly decrease of TH immunoreactivity in PQ + MB LD in the SN and CP was observed, and a significant increase of SNCA immunoreactivity in the PQ + MB LD in the SN and PQ + MB HD in CP were observed. Parkin gene expression showed a decreased statistically significant in SN, no changes were observed for both groups in the CP. No changes in the immunoreactivity of P-ERK ½, CREB and GAD 65/67 were observed in the SN and CP. A significant decrease in the KOP mRNA levels was observed in the SN and CP,

with an increase only in PDYN mRNA levels for PQ + MB HD in the SN. A statistically significant decrease of NOP receptor mRNA levels was observed, together with a significant increase for N/OFQ gene expression in the SN for PQ + MB LD. In the CP, no changes of the N/OFQ-NOP system were detected. These data strengthen the hypothesis that this neuropeptidergic system could be implicated in the mechanisms underlying Parkinson's disease.

Then we studied Alzheimer's disease. This disorder is defined as a progressive neurologic disease of the brain leading to the irreversible loss of neurons and the loss of intellectual abilities, including memory and reasoning, which become severe enough to impede social or occupational functioning. One of the problems of Alzheimer's disease is that symptoms of disease appear to develop only after substantial cell loss has occurred in brain. Effective biomarker tests could prevent such devastating damage occurring. This will be particularly important once a cure or more effective medications become available. We utilized the peripheral blood cells of AD discordant monozygotic twin in the search of peripheral markers which could reflect the pathology within the brain, and also support the hypothesis that PBMC might be a useful model of epigenetic gene regulation in the brain. We investigated the mRNA levels in several genes involve in AD pathogenesis, the protein precursor amyloid (APP), presenilin 1 (PSEN1), sirtuin 1 (SIRT1), PIN1 and Apolipoprotein E (APOE), as well DNA methylation by MSP Real-Time PCR. Finally by Western Blotting we assess the immunoreactivity levels for H3K9 Ac and K3K4 me3 considered as marker of gene activation and H3K27 me3 marker of gene silencing. We found that APP, SIRT1 and PIN1 gene expression was highly up-regulated in the AD *versus* the healthy twin, along with a substantial reduction of H3K9 Ac and H3K4 me3, and an increased immunoreactivity in H3K27 me3. No change in mRNA levels of APOE was observed. We found a decrease in the values of PSEN1 gene expression in the AD twin versus the healthy twin. A general DNA hypomethylation of all gene promoters studied was also observed in both twins. Our study should provide insights into the molecular causes of differential susceptibility to AD in genetically identical organisms and clarify the importance of epigenetic factors in its mediation. Our results support the idea that epigenetic changes assessed in PBMCs can also be useful in neurodegenerative disorders, like AD and PD, enabling identification of new biomarkers in order to develop early diagnostic programs.

Abbreviations

5-HT	5-hydroxytryptamine
6-OHDA	6-hydroxydopamine
Aβ	beta-amiloid
AD	Alzheimer' disease
APOE	Apolipoprotein E
APP	Amyloid precursor protein
CNS	Central nervous system
CP	Caudate putamen
CSF	Cerebrospinal fluid
DDCt	Delta-delta Ct
DNMT	DNA methyltransferases
ERK	Extracellular signal-regulated kinase
ƑAD	Familial AD
GABA	ɣ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HATs	Histone acetyltransferase
HD	Huntington disease
HDACs	Histone deacetylase
L-DOPA	L-3,4-dihydroxyphenilalanine
MAO-B	Monoamine oxidase B
MB	Maneb
MCI	Mild cognitive impairment
Mn-EBDC	Manganese ethylenebis-dithiocarbamate
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP+	1-methyl-4-phenyl-2,3-dihydropyridinium
MZ	Human monozygotic
NFTs	Neurofibrillary tangles
NMDA	N-methyl-$_D$-aspartate
N/OFQ	neuropeptide nociceptin/orphanin FQ
NOP	NOP receptor
PBMC	Peripheral blood mononuclear cells
PD	Parkinson's disease
PIN1	Peptidyl-prolyl *cis/trans* isomerase
ppN/OFQ	Pre-pro nociceptin
PQ	Paraquat
PSEN1	Presenilin 1
PSEN2	Presenilin 2
ROS	Reactive oxygen species
SIRT1	Sirtuin 1
SN	Substantia nigra
SN*pc*	Substantia nigra pars compacta
SNCA	Alpha-synuclein
TH	Tirosyne hydroxylase
UCH-L1	Ub carboxyl-terminal hydrolase
VTA	Ventral tegmental area

1. GENERAL BACKGROUND

1.1 NEURODEGENERATION

With neurodegeneration is intended the progressive loss of structure or function of neurons, including neuron death. Neurodegeneration can be found of many different levels, from molecular to systemic, and several diseases like Parkinson's, Alzheimer's, and Huntington's occur as a result of neurodegenerative processes. Neurodegenerative disorders constitute one of the major challenges of modern medicine. Although these diseases are relatively common and highly debilitating, the physiopathologic mechanisms implicated are poorly understood with the subsequent lack of effective therapies. (Rubinsztein 2006, Bredesen et al 2006).

Recently, linkage studies have begun to identify genes underlying heritable forms of the neurodegenerative disorders. While these breakthroughs potentially provide a window into the more common sporadic forms of these disorders, we currently know very little about the functions of these genes and the mechanisms by which their mutational alteration results in neuronal death. Several studies have shown many similarities between different neurodegenerative diseases suggesting a common therapeutic approach that could be useful for many diseases simultaneously. There are many parallels between different neurodegenerative disorders including atypical protein assemblies as well as induced cell death (Rubinsztein 2006, Bredesen et al 2006).

1.1.2 PARKINSON'S DISEASE

In 1817 James Parkinson, an english surgeon, in his classic monograph "an essay on the shaking palsy?", described the main features of what is now known as Parkinson's disease (PD) giving case histories of six sufferers. PD is second only to Alzheimer`s disease (AD) as the most common idiopathic neurodegenerative disorder affecting roughly 0,5-1% of the current population among 65 to 69 years of age, rising to 1 to 3 percent among persons of 80 years of age and older (Tanner and Goldman 1996).

The first and most prominent manifestations of PD are the impairments in motor function. This neurological disorder has 4 cardinal features: bradykinesia, muscular rigidity, tremor at rest and postural unbalance, causing falls and crawling. Not each patient shows at the beginning classic signs (Zigmond et al 2002). However with disease progression a new set of problems emerges, including speech difficulties, cognitive dysfunction, and depression, sleep disturbance, constipation, bladder and sexual dysfunction, and a series of autonomic problems. Patients with PD shows an increased risk of cognitive and psychiatric diseases, with dementia and depression as the most common ones, but hallucinations, irritability, apathy and anxiety have also been described (Zigmond et al 2002). Without treatment, in 5 to 10 years, PD progresses up to rigidity and akinesia and prevents patients to take care of themselves. Death commonly results from complication associated with immobility, and includes pneumonia or pulmonary embolism.

PD is characterized at neuropathologic level by loss of dopaminergic neurons normally present in substantia nigra pars compacta (SN*pc*). This area provides dopaminergic innervation to striatum (caudate and putamen nucleus) and to globus pallidus (Bohlen und Halbach et al 2004, Schulz and Falkenburger 2004). The disease becomes clinically apparent once ≈ 70% of the dopaminergic neurons of the SN are lost. However, has been demonstrated that neurodegenerative damage is not only restricted to substantia nigra (SN) neurons but can also affect noradrenergic (locus coerulus), serotoninergic (raphe), and colinergic (Meynert basal nuclei and vagus dorsal motor nucleus) systems, cerebral cortex, olfactory bulb and autonomous nervous system. Damage within these areas will explain cognitive and psychiatric signs observed in aged patients, even 10

years before PD diagnosis, suggesting an early damage of this areas respect to dopaminergic damage.

Another neuropathological hallmark of PD is the presence of Lewy Bodies, spheric intracytoplasmatic inclusions, constituted by insoluble proteins deposits, like α-synuclein (SNCA), parkin, ubiquitin, synphilin y neurofilaments, present in the neuronal soma and dendrites of affected neurons. They have a diameter of approximately 15 μm and have an organized structure with a dense nucleo surrounded by a clear halo. Lewy Bodies are found in all affected brain regions (Forno 1996, Spillantini et al 1998) however they are not specific of PD and can be also found in AD**,** Huntington Disease (HD), Dementia and aged healthy subjects. Additionally, not all cells are able to form these protein aggregations. Vagus dorsal nucleus, locus coerulus, SN*pc*, mesocortex and neocortex have been described as the more susceptible areas, but no relation between this histological change and loss of neurons has been found. It has been hypothesized that a mutation could affect protein degradation leading to intracellular accumulation, and damage will be due to interference with cellular traffic and/or kidnap of critical cellular proteins (Schulz and Falkenburger 2004). Thus it has been proposed that dysfunction of protein degradation pathways plays an essential role in the degenerative process observed in PD.

Although diagnosis is made clinically, other disorders can show prominent symptoms and signs similar to PD, such as postencephalite, drug-induced, and arteriosclerosis, situation noted as Parkinsonism and which may be confused with PD until a final confirmation by an autopsy (Hughes et al 1992).

1.1.2.1 PRINCIPAL HYPOTHESIS OF DOPAMINERGIC NEURONAL DEATH

OXIDATIVE STRESS

The brain depends mostly on mitochondrial energy supply which is associated with the production of highly reactive oxygen species (ROS), the 95% of the molecular oxygen is metabolized within the mitochondria by the electron transport chain, so mitochondria are highly exposed to oxidative stress which may induce damage in distinct neuronal populations (Tritschler et al 1994). Mitochondrial dysfunction increases free iron levels and impairs free radical defense mechanism leading to oxidative stress increase. Reduced levels of glutathione in the midbrain may be indicative of increased free radical levels (Sian et al 1991).

Dopamine metabolism can also be a source of ROS in nigral neurons, leading to functional alterations in proteins, lipids and DNA. Lipid damage, in turn, causes loss of membrane integrity and decreased membrane-permeability to ions, which can promote excitotoxicity (Halliwell 1992). Cytosolic dopamine produces electrophilic semiquinones and quinones which themselves act as oxidants by supporting ROS formation (Sulzer and Zecca 2000). It has also been recognized that oxidative stress leading to caspase activation and consequent apoptosis are clearly evident in PD (Friedlander 2003). These findings provide a plausible link between oxidative damage and formation of the Lewy bodie, oxidative damage induce SNCA aggregation and impairs proteosomal ubiquitination and degradation of proteins (Jenner 2003). Mitochondrial dysfunction and oxidative stress might reset the threshold for activation of apoptotic pathways in response to Bax and other pro-apoptotic molecules (Henchcliffe and Flin Beal 2008). Several animal models of PD such rotenone, 2,4,5-trihydroxyphenethylamine or 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), have been shown to have multiple mitochondrial dysfunctions including increased ROS generation and striking sensitivity to stressors (Onyango 2008).

MITOCHONDRIAL DYSFUNCTION

The mitochondrion is the primary site for the generation of energy supply for the cell. The mitochondrial generation of energy is regulated by five respiratory chain complexes. A defect in the mitochondrial complex I could contribute to cell degeneration in PD through decreased ATP synthesis and dearrangements in complex I cause SNCA aggregation, which contributes to demise of dopaminergic neurons (Dawson and Dawson 2003). Dysfunction of the electron transport chain activity in neurons of the SN*pc* might be related to PD induced by environmental toxins (O. von Bohlen und Halbach et al 2004). 1-methyl-4-phenyl-2,3-dihydropyridinium (MPP+), 6-OHDA and rotenone acts as a mitochondrial toxin that selectively inhibits the mitochondrial complex I (Greenamyre et al 1999, Jenner 2001). Mitochondrial dysfunction, however, might not exclusively be related with dopaminergic neurons of the SN*pc* but may be systemic, since mitochondrial dysfunction in PD is also observed in the striatum and other tissues (Parker and Swerdlow 1998).

EXCITOTOXICITY

Damage due to excess of glutamate, which changes the permeability of cells to calcium by acting on and through N-methyl-$_D$-aspartate (NMDA) receptors, is considered to be involved in neurodegeneration. Massive activation of glutamate receptors can result in excessive rises in cytoplasmic Ca^{2+} that are thought to underlie the fundamental processes ultimately leading to neuronal death (Mody and MacDonald 1995). The concept of excitotoxicity has also been applied to PD; studies have demonstrated that parkin (hereditary Parkinson disease PARK2 gene product) regulates the function and stability of excitatory glutamatergic synapses. Dopaminergic nigrostriatal neurons are rich in glutamate receptors and receive and extensive glutamatergic innervation from the cortex and the subthalamic nucleus (Olanow and Tatton 1999). The post-synaptic expression of parkin dampens excitatory synaptic transmission and causes a marked loss of excitatory synapses in hippocampal neurons. Conversely, knockdown of endogenous parkin or expression of PD-linked parkin mutants profoundly enhances synaptic efficacy and triggers a proliferation of glutamatergic synapses. This proliferation is associated with increased vulnerability to synaptic excitotoxicity. The resulting excessive glutamatergic drive could be a source of excitotoxicity in the SN. As

described above, persistent activation of NMDA receptor increases intracellular calcium levels. A role for elevated intracellular calcium in the events leading to cell death in PD is supported by the observation that dopaminergic neurons expressing the calcium-binding protein calbindin may be selectively preserved in PD (Lang and Lozano 1998). A number of studies have shown that ionotropic antagonists of the NMDA glutamate receptor subtypes counteract parkinsonian symptoms or act in synergy with L-3,4-dihydroxyphenylalanine (L-DOPA) in animal models of PD. A key indication that environmental factors may play a role in PD came with the discovery of MPTP, which has been used to develop animal models of PD in which the pathogenesis of the disease and a variety of therapeutic interventions designed to prevent or reduce dopaminergic neurodegeneration can be studied.

PROTEASOMAL DYSFUNCTION

A significant feature of PD pathology is the presence of Lewy bodies. This is indicative of incomplete clearance of the target proteins by the ubiquitin-proteosome system. This may arise from increased protein oxidation products overwhelming the proteolytic capacity of the proteosome (Elkon et al 2004). Lewy Bodies are constituted by insoluble proteins deposits, like SNCA, parkin, ubiquitin, synphilin and neurofilaments, with SNCA as main one.

Alpha-synuclein

Synucleins are a family of 15-20 kDa proteins currently consisting of three diferent members: α-synuclein, β-synuclein and γ-synuclein (Clayton and George 1998). All three different synucleins are expressed in human and rodent brains (Giasson et al 2001).

In cell cultures, using SNCA transfected B103 neuroblastoma cells, has been shown that SNCA up-regulated the expression of calveolin-1 and down-regulates extracellular signal-regulated kinase (ERK) activity (Hashimoto et al 2003). Since ERK plays a central role in several neuronal functions, including survival, neuronal growth, synapse formation, synaptic plasticity and long-term potentiation, modulation of the ERK pathway might be an important mechanism in normal SNCA functions. In addition, SNCA might also be associated with axonal transport (Mizuno et al 2001).

Interestingly, several pesticides, including rotenone and paraquat, can induce a conformational change in SNCA and, thus, accelerate the rate of SNCA fibrils formation in vitro (Uversky et al 2001). The molecular mechanism by which abnormal SNCA oligomers cause dysfunction and death of dopaminergic neurons in the SN*pc* is still unknown. However, it has been confirmed that SNCA co-localizes with tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine biosynthesis (Perez et al 2002). In cell-free systems, a dose-dependent inhibition of TH by SNCA has been observed. Under normal conditions, low concentrations of SNCA might have neuroprotective properties, for the contrary, high micromolar concentration SNCA exerts neurotoxic effects in primary neuronal cultures (Seo et al 2002).

Parkin

Parkin is expressed in neurons, but also in glial cells and blood vessels of the human, monkey (Zarate-Lagunes et al 2001) and rat brain. Also has been detected in neuronal perikarya of the SN, the striatum, the hippocampal formation, the pallidal complex, the red nucleus, and the cerbellum in human and monkey brains (Zarate-Lagunes et al 2001). In rats, parkin mRNA and protein have been detected in the SN*pc* (Horowitz et al 1999). Its ability to enhance the ubiquitination and degradation of misfolded tubulins may play a significant role in protecting neurons from toxins that cause PD (Ren et al 2003). The function of parkin in the ubiquitination pathway is to target misfolded proteins for degradation, as parkin protects against neurotoxicity induced by unfolded protein stress (Imai et al 2000, Chung et al 2001). Additionally, it was found that unfolded protein stress induces up-regulation of both the mRNA and protein level of parkin. Furthermore, overexpression of parkin specifically suppressed unfolded protein stress-induced cell death. In addition, parkin is able to suppres SNCA-induced toxicity in cell culture systems (Oluwatosin-Chigbu et al 2003). The neuronal expression pattern of SNCA and parkin mRNA are highly overlappping in the brain, suggesting that these two proteins may play a role in the pathophysiology of PD (Solano et al 2000). Indeed, parkin has been shown to ubiquitinate Lewy bodies-like inclusions (Chung et al 2001) and to protect against the toxicity associated with SNCA (Petrucelli et al 2003)

1.1.2.2 ETIOLOGY

GENETIC FACTORS

The familial PD can be divided into two types, autosomal dominant and autosomal recessive. Autosomal dominant PD can be caused by mutations in at least two proteins; SNCA and Ub carboxyl-terminal hydrolase (UCH-L1). The SNCA gene encodes a presynaptic protein that may play a role in learning and memory and synaptic plasticity (Dev et al 2003). Two single point mutations in SNCA, A30P and A53T, have been reported (Polymeropoulos et al 1997). On the other hand, UCH-L1 is an enzyme also found in Lewy bodies (Lowe et al 1990), and mutation in this enzyme may then lead to the mismanagement of Ub recycling.

Autosomal recessive young-onset Parkinson is characterised by an early age of onset, beginning in the late 20s and generally before the age of 40, and the clinical feature is indistinguishable from idiopathic PD. It was shown that autosomal recesive young-onset PD has a recessive mode of inheritance where both alleles of parkin are mutated, and is recognized that about 50% of the European family affected by autosomal recessive Parkinsonism have mutations in the parkin gene (Abbas et al 1999, Lücking et al 2000, Kahle et al 2000).

ENVIROMENTAL FACTORS

Genetics forms of PD represent less than 10% of current cases, thus the causes of the vast majority of sporadic cases of PD are still unknown. Accumulating evidence strongly points to environmental toxins as feasible triggers of neurodegeneration of nigrostriatal dopaminergic neurons (Cichetti et al 2005, Meredith et al 2008).

Exposure to air pollution and heavy metals is correlated to PD prevalence (Aquilonius and Hartvig 1986, Gorell et al 1998), and the common use of pesticides in rural life has been correlated to Parkinsonism in humans (Di Monte et al 2002). Moreover, pesticides are often used in overlapping territories leading to exposure to multiple potentially toxic agents, which might act additively or synergistically (Thiruchelvam et al 2000).

1.1.2.3 ANIMAL MODELS OF PARKINSON'S DISEASE

The development of animal models for PD is an important approach to the elucidation of pathogenetic mechanism of neuronal dysfunction and degeneration within the SN underlying movement deficits (Cichetti et al 2005). Neurological disorders in humans can be modeled in animals using standardized procedures that emulate specific pathogenetic events and their behavioral outcomes. Murine models can demonstrate many pathophysiological features of PD and their use has increased our understanding of the mechanism underlying this neurodegenerative disorder (Dauer and Przedborski 2003) and opened doors to exploration of neuroprotective and neurorestorative strategies (Dawson and Dawson 2002).

GENETIC ANIMAL MODELS

- Mouse model based on the deletion of genes important for the development or maintenance of dopaminergic neurons or their phenotype. These mice exhibit dopaminergic cell loss at various times in their life, thus reproducing a cardinal feature of PD (Pitx3 -/- mice and engrailed knock-out mice).
- Mouse or rat models based on expression or deletion of genes known to cause familiar forms of PD (SNCA, Parkin, PINK1, DJ1 and LRRK2).
- Based on virally mediated expression of genes or mutations known to cause familial PD, usually in nigrostriatal dopaminergic neurons.

ENVIROMENTAL ANIMAL MODELS

Neurotoxins

6-Hydroxydopamine

6-OHDA was the first chemical substance discovered that shows a specific neurotoxic effect on central monoaminergic neurons (Ungerstedt 1968). Is a neurotoxin with a structure similar to dopamine and norepinefrine, and using the same catecholaminergic transport system produces specific degeneration of catecholaminergic neurons (Betarbet et al 2002). Within neurons it is rapidly oxidated producing hydrogen peroxide and

paraquinone, both extremely toxic. It is not capable of produce inclusions-like Lewy bodies (Meredith et al 2008). 6-OHDA seems to be toxic to the mitochondrial complex I and induces generation of ROS. It is not able to cross the blood-brain barrier, therefore has to be applied directly into the SN or the striatum. The magnitude of the lesion depends on the amount of 6-OHDA injected, the site of injection and the species used (Betarbet et al 2002). It is usually injected unilaterally, while the intact hemisphere servs as internal control. This unilateral 6-OHDA injection represents the so called "hemiparkinson model" (Perese et al 1989), which is characterized by an asymmetric motor-circling behavior after administration of dopaminergic drugs. However, the 6-OHDA model does not mimic all pathological or clinical features of PD, but induces acute effects, which is different from the slow progresive nature of human PD.

MPTP

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a bypass product of the chemical synthesis of a meperidine analog with potent heroin-like properties. Drug addicts who toke MPTP accidentally developed a syndrome that clinically and pathologically resemble PD (Langston et al 1983). Exposure to MPTP results in a selective degeneration of dopaminergic neurons due the toxic effect of the active metabolite MPP+, which inhibits Complex I of mitochondrial electron transport chain. Unlike 6-OHDA, MPTP is highly lipophilic and crosses the blood-brain barrier, therefore the administration can be done at systemic level or directly in the central nervous system (CNS). Once in the CNS, is metabolized by monoamine oxidase B (MAO-B) within the astrocytes to active metabolite MPP^+. MPP^+ has high affinity with dopamine transporter which facilitates their entry into dopaminergic neurons. MPP+ is also sequestered into synaptic vesicles by the vesicular monoamine transporter, preventing its interaction with mitochondria (Reinhard et al 1987, Liu et al 1992). Loss of dopaminergic neurons depends on treatment performed and varying from 20% with a single dose to 50% with an acute treatment for 4 days (Chan et al 1997). Presence of small inclusions containing SNCA has been determined even after 3 days of treatment (Meredith et al 2002). The treatment of primates or rodents with MPTP represents an animal model which reflects many features of human PD. MPTP can produce an irreversible and severe Parkinsonian syndrome that replicates nearly all features of PD, including rigidity, tremor, slowness of movement and even freezing. A dramatic cell loss is accompanied by an increase in the number of SNCA immunoreactive neurons

located in the SN*pc* and in an increase of SNCA mRNA (Vila et al 2000). The regimen of MPTP administration in mice have been shown to determinate the mode of neuronal cell death in the SN. Chronic regimen causes apoptotic cell death of dopaminergic neurons (Tatton and Kish 1997), whereas an acute regimen causes a necrotic cell death of dopaminergic neuron in the SN (Jackson-Lewis et al l995). However, there are some limitations of the MPTP model. In many cases acute MPTP treatment were performed, which do not mimic the progressive degeneration of nigrostriatal dopaminergic neurons in PD. This can be overcome by a model of chronic MPTP regimens, however, long-term treatment with low doses of MPTP has resulted in recovery of motor deficit once the treatment is stopped (Betarbet et al 2002).

Pesticides

The possibility that pesticides and other environmental toxins are involved in the pathogenesis has been suggested by several epidemiological studies (Seidler et al 1996, Gorell et al 1998). Two of the three widely used animal models of PD employ pesticides. Pesticides may be absorbed by inhalation, ingestion or, in some cases, such as the organophosphates, through the skin, i.e. dermal absorption (Dick 2007). Pesticides is defined as any agent used to kill undesired organisms such as insects (insecticides), snails and slugs (molluscicide), rodents (rodenticide), plants (herbicide) or fungi (fungicide). Pesticides can be categorized in a number of ways, including their acute toxicity to humans, their chemical group or their mode of action. A number of mechanisms by which pesticides might lead to PD has been identified, including mitochondrial dysfunction, oxidative stress, protein aggregation and altered dopamine levels (Dick 2007).

Rotenone

Rotenone is a naturally occurring complex ketone, derived from the root of *Lonchocarpus* species. Originally, rotenone was employed by Indians as a fish poison. One of the biggest advantages of rotenone as a pesticide is that it biodegrades in few days, even if spread over hundreds of acres of agricultural land (Uversky 2004). It has a lipid component which allows easily cross blood-brain barrier (like MPP^+) favoring rapid distribution in the brain; does not depend on dopamine transporter for celular entry and furthermor, is not sequestered into synaptic terminals (Dauer et al 2002).

Once in the brain, its accumulates in subcellular organelles, such as mitochondria (Talpade et al 2000), where it impairs oxidative phosphorylation by inhibiting complex I of the electron-transport chain (Schuler and Casida 2001), microgliosis, increased iron deposits and the formation of inclusion-like Lewy bodies with ubiquitin and SNCA in nigral cells, quite similar to Lewy bodies seen in PD (Meredith eta al 2008). However there is also a debate concerning the specificity of rotenone for the dopaminergic system, also has been demostrated that systemic administration of rotenone produces selective damage in the striatum but not in the SN (Ferrante et al 1997). This raises the questions whether rotenone exclusively acts on mesencephalic dopaminergic neurons, or whether other striatal projection systems are similarly affected (O. Von Bohlen und Halbach et al 2004).

Paraquat

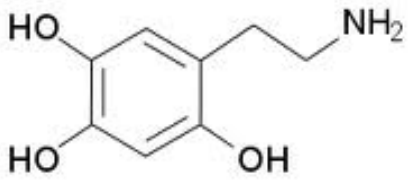
Paraquat (1,1-dimethyl-4,4-bipyridinium) (PQ) is a nonselective bipyridyl contact pesticide that was first produced in 1961. The geographical pattern of usage strongly overlaps with the incidence of idiopathic PD in several countries (Rajput and Uitti 1987, Priyadarshi et al 2001, Di Monte et al 2002). It gained considerable attention because of its extreme toxicity in cases of human exposure, it's know to cause lung, liver, kidney and brain injury (Uversky 2003, Hatcher et al 2008). Is poorly absorbed when inhaled but causes severe illness when ingested orally, usually causing death within 2 days of ingestion of 50 mg/kg. At lower doses, death may be delayed for several weeks. PQ crosses the blood-brain barrier, but slowly, inefficiently, and to a limited extent, although detectable levels of the herbicide have been measured in the CNS after its systemic injection into rodents (Corasaniti et al 1998). PQ is transported into mitochondria by a carrier-mediated process (Cochemé and Murphy 2007), where it is reduced by complex I forming a PQ radical capable oxidatively damaging the mitochondria. Thus, whereas MPP+ and rotenone directly inhibit Complex I function, PQ indirectly disrupts mitochondrial function via intra-mitochondrial ROS formation through complex I interactions with PQ (Meredith et al 2008). These ROS interact with unsatured lipids of membranes (lipid peroxidation) and destroy organelles subsequently leading to cell death (Dodge 1971). In parallel to the neuronal toxicity induced by pesticides and herbicides, the neuroinflammatory response is increasingly suspected to induce more degeneration in the vicinity of targeted neurons (Cichetti et al 2005). The SN*pc* is particularly rich in microglia, the resident immune surveillance cells in the

brain, and patients with idiopathic PD or MPTP-induced PD display localized microglial activation within the SN (McGeer et al 1987, Langston et al 1999). Microglial activation is also found following PQ and Maneb (MB) exposure in rat ventral mesencephalic cultures (Cichetti et al 2005), and in the nigrostriatal pathways of rodents following repeated systemic administration (McCormack and Di Monte 2003, Cichetti et al 2005). Microglial, once activated by neuronal degeneration, release several cytokines, such as tumor necrosis factor - α, interleukin-1β, and interleukin-6, which, in excess, can have detrimental effects on dopaminergic neurons. Systemic intraperitoneal (i.p.) administration of low doses of PQ to adult mice generates specific loss of dopaminergic neurons in the SN with associated decrease in locomotor activity (Brooks et al 1999, McCormack et al 2002) This is coupled with an up-regulation and aggregation of SNCA (Manning-Bog et al 2002). Another line of evidence suggests the importance of the direct interaction between PQ and SNCA. PQ markedly accelerates the in vitro rate of SNCA fibril formation, with the accelerating effects being clearly dose-dependent (Uversky 2003).

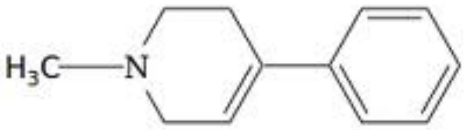
Maneb

MB is a fungicide that inhibits glutamate transport and disrupts dopamine uptake and release (Vaccari et al 1999). The major metabolite active of MB is manganese ethylenebis-dithiocarbamate and has been demonstrated in rats and cell culture systems that MB inhibited mitochondrial function, complex III in particular, and increased oxidative stress. Furthermore, decreased proteosomal function and formation of cytoplasmic inclusions immunoreactive for aggregations of SNCA protein were detected (Zhang et al 2003, Zhou et al 2004, Barlow et al 2005). MB is able to cross the blood-brain-barrier where they may cause selective nigrostriatal neurodegeneration. Humans exposed to MB show signs of parkinsonisms like tremors and slowed movement and gait, developing after years of unprotected handling of exceptionally large amounts of this compound. MB no generate any effects on the serotonergic nerve system like concentration of aspartate, glutamate, taurine or ɣ-aminobutyric acid (GABA) in corpus striatum or in the rest of the brain, indicating that neither the glutammatergic not the GABAergic nerve system was affected by the MB exposure (Nielsen et al 2006). In rodent models, MB is capable of altered behaviour function, inhibit locomotor activity and aggressiveness, rats exposed to MB produced dose-dependent signs of decreased movement, disturbance of coordination, lack of appetite,

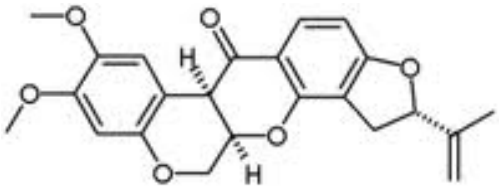
and general weakness (Morato et al 1989). Generally is co-administered with PQ subchronically generating degeneration of nigrostriatal dopaminergic system in mice, suggesting a synergistic effect (Thiruchelvam et al 2000). Studies in older rats have shown that they are very sensitive to the toxic effects of the combination PQ + MB at the same doses used in younger mice (Saint-Pierre et al 2006). Loss of dopaminergic neurons, motor impairment and microgliosis, which are found in both young and old rats, mimic different stages of clinical PD. However, a potential disadvantage of PQ + MB treatment for older rats is systemic lung toxicity, which can be lethal. The lungs of animals treated with PQ + MB present alveolitis and/or bronchiolitis with no evidence of bronchitis, lymphoid aggregates, bronchiectasis, or fibrosis. Reduction of the alveolar airspace due to hyperplasia of the alveolar lining cells may have in part led to subsequent respiratory problems (Saint-Pierre et al 2006, Cichetti et al 2005).



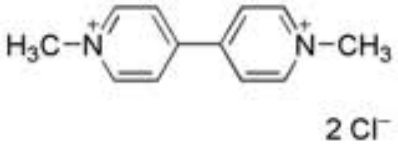
6-OHDA



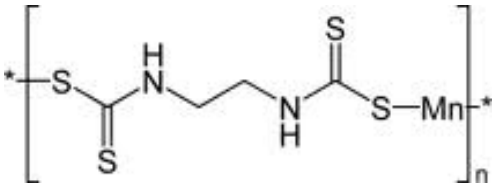
MPTP



ROTENONE



PARAQUAT



MANEB

Fig 1.- Structure relative to 6-OHDA, MPTP, Rotenone, PQ and MB.

1.1.2.4 NOCICEPTIN/ORPHANIN FQ – NOP SYSTEM

NOCICEPTIN RECEPTOR NOP

Two different research groups, by means of molecular biology techniques, have shown in a short period of time (1994-'95) the presence of gene sequences coding for a 370 amioacids proteine, showing primary structure with 7 transmembrane domains receptor (Chuang et al 1996, Meunier et al 2000) (Fig.2). This receptor, which genes have been identify in mouse, human (Mollereau et al 1994) and rat (Chen et al 1994), even if it shows a strong analogy of aminoacidic sequence with diverse types of opioid receptors is unable to interact effectively with ligands of these receptors, such diprenorphine (Dunnill et al 1996). The alingment of aminoacidic sequences of the "orphan receptor" with the μ, δ e k opioid receptors have showed over 80% match for the 2°, 3° and 7° transmembrane domain and little lower for the cytoplasmatic loops; homology level is much lower for N-terminal fraction as well as for the 2° and 3° extracellular loops.

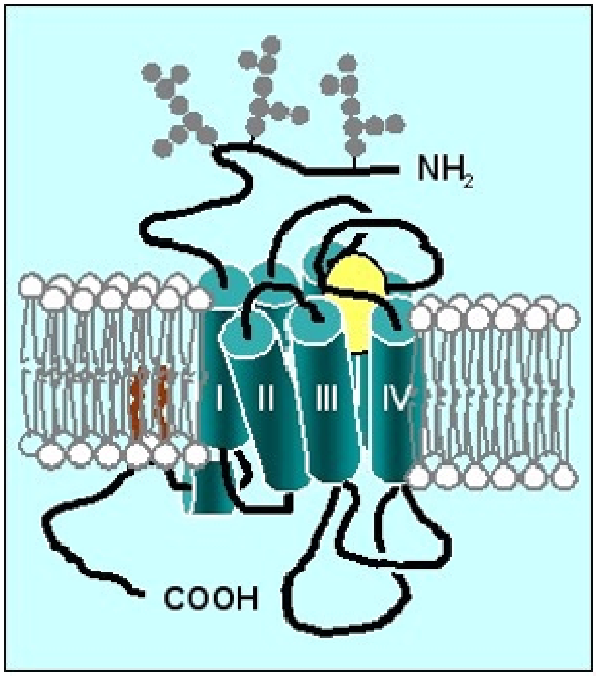


Fig 2.-Schematic representation of tridimensional model of NOP receptor. Scheme has been done using SETOR(4 bis) software.

Although sequence differences between several forms of NOP receptor isolated from different species, there still close to 90% homology between them. Studies focused in receptor distribution have shown presence not only in the CNS (brain and spinal cord) but also in some peripheral tissues; particularly intestine, vas deferens, liver and spleen. No presence has been reported in scheletric muscle, esophagus, kidney, testis and adrenal glands (Wang et al 1994). About CNS distribution, in situ hybridization studies have revealed higher mRNA concentrations in the hypothalamus, amygdala, piriform cortex, dorsal raphe and nucleus coeruleus. Receptor is also present in the cortex, thalamus, hippocampus, periaqueductal grey matter and spinal cord (Fukuda et al 1994, Bunzow et al 1994, Wick et al 1994, Lachowicz et al 1995).

Nevertheless the already mentioned orphan receptor inability to join opioid ligands, there is an interesting functional homology with classics μ, δ e k receptors. Indeed, potent opioid agonist etorphine is able to decrease cAMP accumulation induced by forskolin in Chinese hamster ovary cells stably transfected with the NOP coding gene (Mollereau et al 1994).

Overall, these data have suggested two main considerations:

i) Orphan receptor is an inhibitory G protein coupled receptor for adenylate cyclase and;

ii) Endogenous opioids do not represent the physiologic ligands for this receptor.

THE NEUROPEPTIDE NOCICEPTIN/ORPHANIN FQ (N/OFQ)

In 1995, two independent research teams were able to isolate an endogenous agonist for the orphan receptor. Meunier et al. (Meunier et al 1995) used successive chromatographic purifications of an acid extract of rat brain, while Reinscheid et al. 1995 used suine hypothalamus as starting material.

To highlight active biological compounds within chromatographic fractions both groups have used the same strategy, consisting in the biological dosage of cAMP accumulation inhibition in cells expressing the “orphan receptor”. The following sequencing of this

biological compound showed structural corrispondence with a heptadecapeptide analog to opioid peptides already note, and due a possible nocive effect after intraventricular brain injection in mouse (Meunier et al 1995) or as orphanin FQ (Reinscheid et al 1995) was named nociceptin (N/OFQ). Beyond structural analogies with opioid peptides, an important characteristic is the absence of the tyrosine amino acid in position 1 (unlike all opioid peptides) but a phenylalanine.

Studies carried out to investigate the structure activity relationship of N/OFQ amino acid sequence have indicated that N/OFQ retains full ability to bind with the receptor, whereas the affinity drops drastically with N/OFQ fragments (1-12) and (1-8) (Dooley and Houghten 1996, Dunnill et al 1996). Similar results were obtained in vitro regarding the biological activity of these fragments (Calo et al 1996). Unexpectedly, also the N/OFQ 7-18 and 13-18 shows a high affinity for the NOP receptor (Meunier et al 2000, Butour et al 1997). The all set of observations indicates that the significant portion of N/OFQ is its very basic inner portion, not only in terms of recognition but also in the activation of its receptor.

Additionally, this suggests that although the structural homology of NOP with opioid receptors and opioid peptides withN/OFQ, the latter doesn't seem to bind to its receptor in the same way that dynorphin A binds opioid receptors. In particular, the evidence of an affinity of N/OFQ (Meunier et al 2000, Chen et al 1994, Calo et al 1996) for NOP indicates that the theory of "message-address" previously used for opioid peptides, may not be applicable for the N/OFQ (Chawkin and Goldstein 1981).

At CNS level, distribution of this new heptadecapeptide seems to be more limited than that of its receptor. The N/OFQ is present among others in the preoptic area, septum, amygdala, and median eminence. Instead, low levels of the peptide seem to be present in many areas in which the receptor is highly expressed, such as cortex, nucleus suprachiamaticus, supraoptic nucleus, paraventricular and ventromedial hypothalamus and the dorsal raphe (Watson et al 1996). Finally, elevated levels were found in the superficial layers of the dorsal horn of the spinal cord, trigeminal complex and other areas involved in pain transmission such as the periaqueductal gray matter (Schulz et al 1996).

Pharmacological Effects of Nociceptin/Orphanin FQ

The responses evoked by N/OFQ at the cellular level are similar to those obtained by other receptor agonists, members of the 7-transmembrane domain Gi / Go proteine coupled superfamily. However, it should be noted that in the absence of antagonists against the orphan receptor, the actions of N/OFQ alone, attributed to its interaction with NOP, are only those observed in cells transfected with this receptor while those in non-transfected neuronal tissues are still to be considered only as probable. In transfected cells, N/OFQ strongly inhibits adenilylociclase (Meunier et al 1995, Reinscheid et al 1995) stimulated by forskolin and determines the opening of the "inward rectifier" K + channel (Matthes et al 1996). This action on K + channels was also observed in the dorsal raphe, locus coeruleus and periaqueductal gray matter (Wu et al 1997, Connor et al 1996, Vaughan and Christie 1996).

The neuropeptide is also able to inhibit the entry of Ca^{2+} through voltage-dependent channels in cell cultures (SH-SY5Y) and in cultured hippocampal pyramidal neurons (Connor et al 1996_b). Inhibition of Ca^{2+} voltage-sensitive channels at hippocampal level, involves G_i proteins since it is prevented by pertussis toxin pretreatment and involves multiple types of Ca^{2+} channels (N-type, L and P / Q).

Always at CNS level, has been observed that N/OFQ inhibits, through mechanisms currently not clarified, the release of glutamate and GABA from nerve endings (Vaughan and Christie 1996, Knoflach et al 1996). Reggarding peripheric system, N/OFQ is able to block the release of acetylcholine caused by light stimulation in the retina of rabbit (Nicol et al 1996). In the sympathetic nervous system, this neuropeptide is able to effectively block neuromuscular transmission in the vas deferens of rat, rabbit (Neal et al 1997) and mouse (Nicholson et al 1996, Calo et al 1996). While, in the parasympathetic system, some results indicate (Berzetei-Gurske 1996) that N/OFQ is able to partially inhibit release of acetylcholine from nerve endings in the trachea. In fact, the same investigation that led to the isolation and characterization of this peptide (Meunier et al 1995, Reinscheid et al 1995), has also show that intracerebroventricular N/OFQ administration in mice, causes a lowering of the threshold in both tests hot plate and "tail-flick" (Reinscheid et al 1995). Moreover, the nature of the pro-nociceptive neuropeptide seems further confirmed by the observation that by reducing the expression of the orphan receptor, by administration of antisense oligonucleotides for

NOP, the opposite effect is obtained, namely an increase in nociceptive threshold (Calo et al 1996).

However, with the progress of the studies, the overall picture of the actions induced by N/OFQ in nociceptive transmission has revealed more complex. Some authors have suggested the involvement of stress-induced autoanalgesia mechanisms in N/OFQ action (Patel et al 1997). Other studies have also suggested the existence of a biphasic response comprising hyperalgesia followed by a period of analgesia (Mogil et al 1996). The action exerted by N/OFQ in the spinal cord is still controversial. In fact, some authors did not observe significant alterations of the nociceptive threshold after intrathecal administration of N/OFQ in the rat (Rossi et al 1996), while other investigations have revealed an inhibitory effect of the neuropeptide on synaptic responses of type C fibers (Tian et al 1997, Faber et al 1996).

The effects on locomotor activity evoked by the neuropeptide are still controversial. While some groups have observed a decrease, some data suggest an increase in locomotion following intracerebroventricular administration of low doses of peptide (Giuliani and Maggi 1996).

Regarding the motivational activities, N/OFQ doesn't seem to induce reward or adverse effects (Florin et al 1996), although nucleus accumbens has been shown to contain high levels of mRNA for its receptor and that the peptide is able to decrease the release of dopamine in the same nucleus (Devine et al 1996). It is known that nucleus accumbens is involved in several functions such reinforcement, drug reward and locomotor behavior (Murphy et al 1996). Recent results have suggested that N/OFQ suppresses the activity of the dopaminergic reward pathways of the mesocortic-limbic system not producing, as already mentioned, neither preference nor aversion in the test of conditioned choice (place preference), but recent studies on the same test, indicates that N/OFQ has been shown capable of modifying, in an inhibitory manner, conditioned preferences indiced by other substances, such alcohol and morphine (Devine et al 1996_b). Where recently observed that chronic use of high doses of morphine accelerates the release and biosynthesis of N/OFQ in the rat brain to antagonize the effect of opioids. This effect seems to play a role in the development of tolerance to morphine. It has been suggested that N/OFQ may serve as a retarded negative feedback control for

opioid analgesia (Murphy et al 1999, Yuan et al 1999). Some results indicate that N/OFQ decreases the activity of dopaminergic mesocortico-limbic neurons through an action in the ventral tegmental area (VTA). This effect is not only transient but demonstrates rapid tolerance and thus is insufficient to prevent the development of sensitization to cocaine. The ability of the peptide to induce sensitization to cocaine when administered alone, despite its acute inhibitory effects, is unique and requires further studies to elucidate the responsible mechanisms (King et al 1998). Behavior reinforcement influenced by the nucleus accumbens and the hypothalamus is that of nutrition. It 'was shown a definite increase in the effect of the central rewarding drugs of abuse in animals subjected to food restriction (Narayanan and Maidment 1999).

More recently it has been shown that administration of N/OFQ in the ventromedial hypothalamic nucleus and the nucleus accumbens increased food intake in rats (Cabeza De Vaca and Carr 1998). The role of N/OFQ in stimulating the nutrition behavior is supported also by a recent study showing a reduction of hyperphagia induced by N/OFQ, following central administration of antisense probe against NOP (Stratford et al 1997). As pointed several times by several authors there is a need for new molecules, possibly non-peptide, which selectively activate (agonists) or block (antagonists) the NOP receptor. In order to demonstrate conclusively that the effects of N/OFQ are produced by its interaction with the NOP receptor, is more useful to have pure and selective antagonists for the NOP receptor. To do this, right now, are mainly used molecules that can antagonize the NOP receptor, although none has been shown to have pure selectivity and antagonistic properties as needed to provide clear results. These compounds are:

1. Naloxone benzoilidrazopne (NalBzOH), a non-selective opioid receptor ligand was observed to be able to competitively block some effects of N/OFQ, with low power (pA2≈6.0-6.5) (Matthes et al 1996, Siniscalchi et al 1999, Calo' et al 2000). However, this drug is not ideal because it has also affinity for κ and μ opioid receptors, and exerts in these receptors a mixed agonist-antagonist action, particularly acts as an agonist for μ receptor. This effect can be antagonized by the presence of naloxone, however, the use of that substance may result inappropriate in studies on the pharmacological effects of N/OFQ, since although the low affinity for the NOP receptor, in some in vivo studies

has proved to be an antagonist indirect effects produced by N/OFQ (Schlicker et al 1998).

2. [Phe^{1}ψ(CH_2-NH_2)Gly^2]nociceptin(1-13)NH2, also known as [F/G]NC(1-13)NH_2, a selective pseudopeptide for the NOP receptor, even when selective for δ and κ are not for μ, because its selectivity factor against this receptor is less than 100 (Guerrini et al 1998). This compound was seen to behave as a selective antagonist of the NOP receptor in an in vitro study on mouse vas deferens and guinea pig ileo electrically stimulated (Paul et al 1990). However, since then, many studies, both in vivo and in vitro have been published on the action of this pseudopeptide, showing that it actually can act as an antagonist, partial agonist (Varani et al 1999) or full agonist depending on the preparations.

3. The [Nphe^1]nociceptin(1-13)NH_2, a recently discovered peptide that seems to have shown activity as a pure antagonist in several in vivo and in vitro studies (Sbrenna et al 2000, Siniscalchi et al 1999). However, recent in vivo studies have produced conflicting results, they see such a compound to act as a partial agonist.

The J-113397, a potent antagonist selective for the NOP receptor, has a chemical structure is structurally unrelated to N/OFQ (Calo' et al 2000).

INTERACTION OF NOCICEPTIN/ORPHANIN FQ-NOP RECEPTOR SYSTEM WITH OTHER NEURONAL SYSTEMS

Noradrenergic system

Many studies have shown that N/OFQ inhibits the release of noradrenaline in slices of cerebral cortex in rat, mice and guinea pigs (Rizzi et al 2002, Kawamoto et al 1999, Siniscalchi et al 1999), and in slices of rat hippocampus and hypothalamus (Schlicker et al 1998). This is consistent with the distribution of the NOP receptor, which is expressed in various brain regions rich in noradrenergic fibers such as the hippocampus, tract of the solitary nucleus and lateral reticular nucleus (Werthwein et al 1999).

In particular, the presence of the NOP receptor in some hippocampal formations suggests a possible involvement of the NOP receptor in cognition and memory.

Dopaminergic system

In vitro studies on slices of cerebral cortex in mice have demonstrated that N/OFQ inhibits the release of dopamine (Siniscalchi et al 1999). On the other hand, in vivo studies have demonstrated that N/OFQ administered intracerebroventricularly (Devine et al 1996) or by microdialysis, directly into the ventral tegmental area (Mollereau and Mouledous 2000) reduce the release of dopamine in the nucleus accumbens of anesthetized rats. Additional in vivo studies have shown that N/OFQ, administered intracerebroventricularly, is not able to modify the release of dopamine in the nucleus accumbens and the caudate nucleus, but prevent the increase of dopamine release, caused by morphine administered systemically, both in the nucleus accumbens and in the caudate nucleus (Murphy and Maidment 1999, Di Giannuario et al 1999). The inhibition of mesocortico-limbic dopaminergic fibers by N/OFQ agrees with various experimental observations that N/OFQ is involved in reward, cognitive and motility mechanisms, which originates right in the mesocortico-limbic system (Werthwein et al 1999). Finally, different in vivo studies have demonstrated that N/OFQ, administered through microdialysis directly into the striatum of naloxone-sensitive unanesthetized rats, is able to increase dopamine release (Di Giannuario and Pieretti 2000).

This facilitator role of N/OFQ on nigro-striatal dopaminergic neurons is in agreement with studies of functional distribution of the NOP receptor, which have evidenced it expression in the nigro-striatal projections involved in the coordination of movement (Werthwein et al 1999).

Serotoninergic system

The N/OFQ is able to inhibit the release of 5-HT (5-hydroxytryptamine), 5-HT, both from synaptosomes (Varani et al 1999) and cortex slices of rats and mice (Sbrenna et al 2000, Konya et al 1998, Maneuf et al 1999). These findings have been correlated with the anxiolytic action of which the NOP receptor and N/OFQ appear to be responsible (Varani et al 1999).

Endogenous opioid system

From a molecular approach, based on genetic criteria, there is no doubt that the NOP receptor and opioid receptors belong to the same family and, additionally, also the precursors of their endogenous ligands (N/OFQ and opioids peptides) have a high homology in genetic profile. However, at functional level the NOP receptor is not an opioid receptor: does not bind opioid receptor ligands with high affinity and, more importantly, pharmacological effects mediated by it are mostly insensitive to the opioid receptor antagonists.

The marked structural analogy between the NOP receptor and opioid receptors (especially κ receptor) and between N/OFQ and opioid peptides (especially dynorphin A) is not reflected on the anatomy, because N/OFQ and opioid peptides appear to be localized in separated neuronal circuits. Moreover, N/OFQ shares with opioid peptides the same pathway of signal transduction mediated by G proteins, in particular both stimulate currents of K^+ "outward" and / or inhibit Ca^{2+} voltage-dependant channels, through which reduces the synaptic efficacy. However, although this is the same signal transduction pathway of opioid receptors, it can lead to opposite effects. In fact, morphine, by suppression of the tonic inhibition exerted by GABA in the ventral tegmental area on mesocortico-limbic dopaminergic fibers, decreases the activity of these neurons, while N/OFQ is able to antagonize this effect by increasing the activity of GABAergic neurons (Sbrenna et al 1999, Ciccocioppo et al 2000, Meunier 1997). So, even when the genetic profile of the nociceptin/NOP receptor belongs to the same family of opioid receptors, under the functional pharmacological profile acts as an anti-opioid system.

GABAergic system

The N/OFQ is unable to modulate the release of GABA in synaptosomes of rat cerebral cortex (Meunier 1997). However, in vivo studies have shown that it is able to increase levels of GABA in the ventral tegmental area (Mollereau and Mouledous 2000). This is correlated with the ability of N/OFQ to decrease the release of dopamine in the nucleus accumbens when administered via microdialysis in the ventral tegmental area. In fact, GABAergic and glutamatergic neurons within the ventral tegmental area are able to modulate in an inhibitory manner the dopaminergic fibers projected from the ventral

tegmental area to the nucleus accumbens (Mollereau and Mouledous 2000, Di Giannuario et al 1999).

It is possible to say that NOP receptor is present in brain areas which are not associated with individual physiological functions or individual neurotransmitter systems, but in areas involved in various processes, such as pain and sensory perception, memory, stress, motility, endocrine regulation and gratification. This wide distribution reflects the involvement of the NOP receptor with a large number of physiological responses, or, more probably the contribution of the NOP receptor system, as well as other systems, to homeostasis through modulation of different neuronal circuits simultaneously. This may explain why the deletion of the NOP receptor gene in mice (knock-out mice) has only a slight impact (Meunier 1997) on the mentioned physiological functions and also why the pharmacological effects of N/OFQ are sometimes contradictory.

Glutamaergic system

Glutamate is the major excitatory neurotransmitter in the central nervous system where it acts upon ionotropic. Although, glutamate plays a central role in excitatory neurotransmission alterations in glutamate homeostasis can have significant repercussions on neurons through the generation of neurotoxic or exitotoxic cascade (Olney 1990). It is believed that excitotoxicity can damage neurons directly through the overstimulation of NMDA receptors as a result of increased release of extracellular glutamate or a reduction in its removal from the synaptic cleft, thus propagating the influx of calcium (Caudle and Zhang 2009). In vitro studies have shown that N/OFQ inhibits glutamate release in cortex slice of rats (Knoflach et al 1996), but is not able to modulate it in synaptosomes derivated from the same brain area (Maneuf et al 1999). On the contrary, in vivo, N/OFQ increases the release of glutamate in the ventral tegmental area, and it has been correlated with the results obtained from similar experiments with dopamine in the nucleus accumbens and ventral tegmental area with GABA.

NOCICEPTIN/ORPHANIN FQ – NOP SYSTEM AND PARKINSON'S DISEASE

The opioid-like neuropeptide N/OFQ and its receptor NOP are widely expressed in cortical and subcortical areas (Darland et al 1998), and particularly in the SN*pc*, a brain area containing dopamine neurons that degenerates in PD, in which 50% of dopaminergic neurons express NOP mRNA, and 50-60% of N/OFQ neurons express glutamic acid decarboxylase mRNA, suggesting that N/OFQ is released from SN*pc* GABA neurons. Endogenous N/OFQ facilitates nigral glutamate release and inhibits nigrostriatal dopamine transmission and motor behaviour (Marti et al 2005). N/OFQ is a 17-amino-acid neuropeptide with high sequence homology with dynorphin A (Witta et al 2004), is derived from a larger precursor, pre pro-nociceptin/orphanin, that is also precursor for several other peptides, including nocistatin, nociceptin-2 and pN/OFQ. N/OFQ has selective affinity for the NOP receptor with negligible affinity for the mu, delta or kappa opioid receptors. N/OFQ expression is strongly induced in neurons and astrocytes by oxidative stress and proinflammatory mediators (Buzas et al 1999). At cellular level, N/OFQ induce an inhibition of adenylate cyclase, activation of the MAP kinase pathway, inhibition of N-type Ca2+ channels and an increase of K+ conductases (Connor et al 1996). Generally the distribution of N/OFQ immunoreactivity in human post-mortem brain is correlated with the distribution of N/OFQ-immunorectivity in rat brain (Witta et al 2004). These suggest that many functional roles for N/OFQ that have been proposed based on studies in rat are probably conserved in human brain.

Several studies have shown that exogenous N/OFQ inhibits activity of dopaminergic neurons in the SN*pc* *in vitro* and nigrostriatal dopaminergic transmission *in vivo* (Marti et al 2004) and elevates the glutamate release in the SN reticulate *in vivo* (Marti et al 2002). It is also know that NOP receptor antagonists facilitate nigrostriatal dopaminergic transmission and motor behavior and inhibits release of glutamate in the substantia nigra reticulate (Marti et al 2002, Marti et al 2004), by depressing an N/OFQergic tone. PD may represent a specific clinical indication for NOP receptor antagonist usage. The 6-OHDA lesion was also associated with reduction of NOP receptor expression in the SN*pc* and, to lesser extent, in the SN reticulate. Because 75% of NOP receptor expressing neurons in the SN*pc* are TH positive (Norton et al 2002), reduction of NOP receptor mRNA levels may reflect loss of dopaminergic neurons (Marti et al 2005). In addition to sustaining parkinsonian-like symptoms, endogenous

N/OFQ also contributes to MPTP toxicity. Indeed, MPTP-treated ppN/OFQ-/- mice displayed higher number of surviving TH-positive cells in SN and fibers in the caudate putamen (CP). N/OFQ, but not other products of the ppN/OFQ gene (nocistatin and N/OFQ II), potentiated the excitotoxic white matter lesions induced by ibotenate via NMDA receptor activation (Laudenbach et al 2001). Glutamatergic mechanisms have been implicated in MPTP toxicity. Indeed, MPP+ inhibits mitochondrial complex I, resulting in a loss of intracellular ATP and generation of ROS, which contribute to degeneration of dopaminergic neurons. Moreover, loss of ATP ultimately causes a fall in neuronal membrane potential, leading to impaired calcium homeostasis and enhanced sensitivity to glutamate mediated excitotoxicity (Nicotra and Parvez 2002).

1.1.3 ALZHEIMER'S DISEASE

In 1911, Alois Alzheimer described a neuropsychiatric disorder affecting the elderly, which is widely known today as AD. AD is the most common irreversible, progressive cause of over 50% of all dementia and actually affects more than 24 million people worldwide. Moreover, over 5 million new cases of AD are reported each year, and the incidence increases from 1% between the ages of 60 and 70 to 6% to 8% at the age of 85 years or older and is likely to increase as a greater proportion of the population ages. The duration of disease is typically 8 to 10 years, with a range from 2 to 25 years after diagnosis. AD is characterized by a severe, progressive memory and cognitive skills loss, accompanied by specific neuropathological changes such as the formation of neurofibrillary tangle and senil plaque, as well as a reduction in levels of acetylcholine. Until today no treatments have been found able to completely stop or slow the progression of this disorder, and the autopsy represents the main mean by which the definitive diagnosis is made (Perl 2010).

MACROSCOPIC CHANGES

AD is a disease of the brain affecting especially temporal and parietal cortex, hippocampus, and amygdala. Most cases of AD shown a modest degree of cerebral cortical atrophy but can also involve the frontotemporal association cortex; additionally the loss of brain tissue generally leads to a symmetrical dilation of the lateral ventricles (Fig 3).

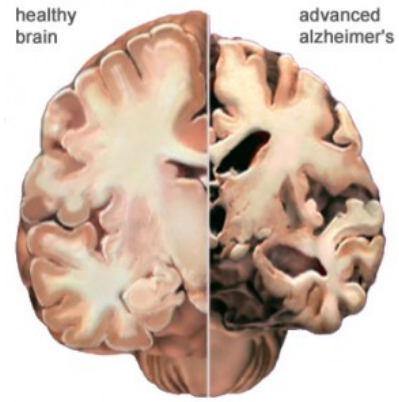


Fig 3.- View of massive cell loss changes the whole brain in advanced AD. Damage areas involved are cortex, hippocampus and ventricles.

MICROSCOPIC CHANGES

Neurofibrillary tangles (NFTs)

Alois Alzheimer noted the presence of abnormal fibrous inclusions whithin the perikaryal cytoplasm of pyramidal neurons, this inclusion are considered a cardinal microscopic lesion associated with the disease. The NFTs appear as parallel, thickened fibrils that surround the nucleus and extend toward the apical dendrite, are composed of abnormal fibrils measuring 10 nm in diameter that occurs in pairs and are wound in a helical fashion with a regular periodicity of 80 nm (Kidd 1963). The primary constituent of the NFTs is the microtubule-associated protein *tau*. The *tau* is abnormally phosphorylated with phosphate groups attached to very specific sites on the molecule (Lee et al 1991). Another constituents associated with the neurofibrillary tangle, are ubiquitin, cholinesterases, and beta-amyloid 4, but *tau* is considered to be the critical constituent of most of these structures. The NFTs are not specific feature of AD, other disorders include postencephalitic parkinsonism, post-traumatic dementia or dementia pugalistica, type C Niemann-Pick disease, and amyotrophic lateral sclerosis/parkinsonism dementia complex of Guam.

Senil plaques

Senil plaques also named neuritic plaques, are complex structures that are defined by the presence of a central core accumulation of a 4 kD protein with a beta-pleated sheet configuration called beta-amyloid (Aβ) (Masters et al 1985, Kang et al 1987). The Aβ peptide is derived from the proteolysis of the amyloid precursor protein (APP) (Glenner and Wong 1984) and correspond a 40 to 43 amino-acid proteolysis product of a larger precursor protein. The deposits of amyloid fibrils were localized extracellularly in the brain. The activity of β- and γ-secretase is required to generate Aβ. Once generated, Aβ is immediately secreted into the media of cultured cells or biological fluids such as plasma and cerebrospinal fluid (CSF) (Haass and Selkoe 1993). The third secretase, the α-secretase, cleaves in the middle of the β-amyloid domain and thus prevents Aβ generation.

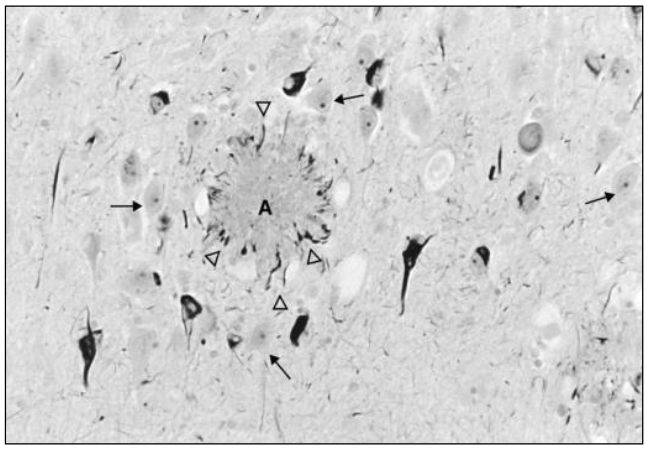


Fig 4.- The classical histopathological lesions of AD demonstrated by the modified Bielschowsky silver stain. In the center, a senile plaque consists of a large, compacted deposit of extracellular amyloid (A) surrounded by a halo of dilated, structurally abnormal, or dystrophic, neurites (open arrowheads).

1.1.3.1 HYPOTHESIS OF ALZHEIMER DISEASE

CHOLINERGIC HYPOTHESIS

It was the first theory proposed by Bartus et al 1982 and is based on the finding of a loss of cholinergic activity in the brain of AD patients (Davies and Maloney 1976, Perry et al 1981). Previous findings supporting this theory into question and it is no longer widely believed that the cholinergic depletion alone is responsible for causing AD. Several studies in humans and non-human primates have suggest a role for acetylcholine in learning and memory, and have reported that blocking central cholinergic activity with scopolamine, young subjects would demonstrate memory deficits similar to those seen in aged individuals. Davis et al, 1999, examined cholinergic markers in the brains of aged human subjects with mild cognitive impairment (MCI) and compared them to AD patients with severe late stage AD; the conclusion of this work was that colinergic dysfunction does not occur until later in the disorder.

AMYLOID HYPOTHESIS

This hypothesis has been described for the first time in 1992 (Hardy and Higgins 1992). This hypothesis also called *"amyloid cascade hyphotesis"* synthesizes histopathological

and genetic information (Fig 5), and claims that the deposition of the Aβ peptide in the brain parenchyma initiates a sequence of events that ultimately lead to AD dementia (Karran et al 2011). This hypothesis does not consider the interaction of Aβ and *tau*. A key role in Aβ production is played by an altered proteolitic cleavage, operated by different enzyme complexes, of the APP. APP cleavage results from sequencially proteolitic action exerted by α, β, γ-secretases, metallproteins able to cut protein at specific points. Under physiologic conditions, APP can undergo two processing ways: non amylodogenic (through α-secretasi) and amyloidogenic (through β-secretasi) with the last inducing production of amyloid peptides of different length. Specifically, in the amyloidogenic way, the proteolitic cleavage by β-secretasi (an aspartil-protease, named BACE, β site-APP cleaving enzyme) at extracellular side is done at N-terminal portion of the Aβ sequence, inducing the formation and secretion of the sAPPβ fragment and the formation of a second one named C99 which remains in the membrane where is subsequently cleaved by γ-secretasi. The end point is the formation and secretion of Aβ fragments, about 39 to 43 amino acids length, into endocytic compartments from where in turn will be exported outside to form the charasteristic plaques.

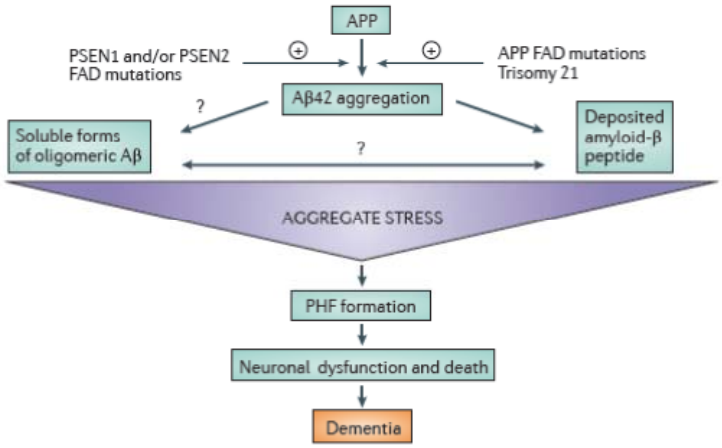


Fig 5.- The amyloid cascade hypothesis. The amyloid cascade hypothesis posits that the deposition of the amyloid-β peptide in the brain parenchyma is a crucial step that ultimately leads to AD. Autosomal dominant mutations that cause early onset familial AD occur in three genes: presenilin 1 (*PSEN1*), *PSEN2* and amyloid precursor protein (*APP*).

TAU HYPOTHESIS

The discovery by Alois Alzheimer of NFTs in the brain of patients with AD provided the basis for a significant amount of studies to elucidate the molecular, cellular and genetic features of this disease (Maccioni et al 2001). However, the discovery that the protein components of NFTs and the paired helical filaments were hyperphosphorylated forms of *tau* was achieved only during the 1980s **(**Kosik et al 1986). *Tau* is a normal constituent of neurons and corresponde to a soluble protein which join to cytoskeletal tubulin to promote assembling and stabilization of neurons (Maccioni and Cambiazo 1995) (Fig 6). In AD as well as in other Taupathies, *tau* protein is abnormally phosphorilated. The hyperphosphorylation of *tau* protein could be related to unbalance kinase (GSK 3β and CDK5) or phosphatase activities, which are further regulated by other proteins like PIN1 (Wang et al 1995, Sze et al 2004), and alters *tau* affinity for tubulin and conferring an increased tendency to form insoluble fibrillar aggregates that are deposited inside cells, compromising synaptic and neuronal functions. The altered phosphorylation pattern of *tau* is evident from the initial stages of the neurodegenerative process, first in the hyppocampus and then in the amygdala and brain cortex. The main effect induced by neurofibrillar aggregates is the modification of neuronal cytoskeleton with loss of cellular organization and alteration of axonal transport preventing the propagation of nerve impulses (Mudher and Lovestone 2002).

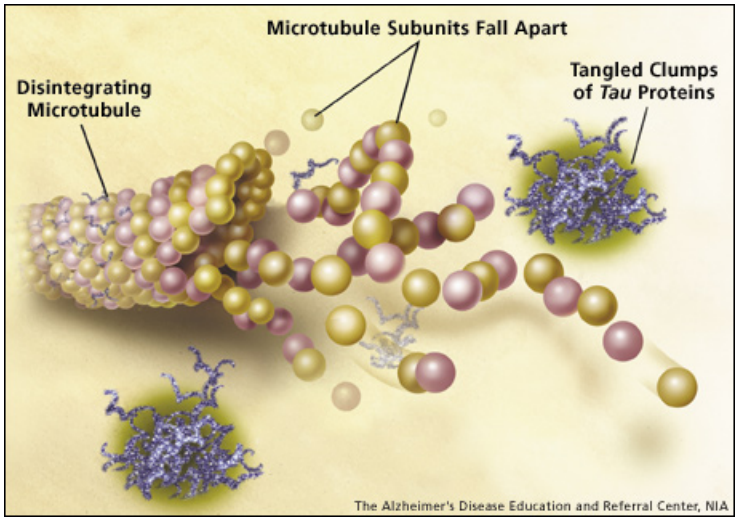


Fig 6.- Tau neurofibrillar aggregates.

1.1.3.2 ETIOLOGY

Both, environmental and genetic factors are involved in AD etiology, but the key pathogenic events leading to neuronal degeneration and dementia, are not yet fully understood.

GENETICS FACTORS

The majority of cases of AD correspond to the sporadic form of this disorder. Approximately 5-10% of patients shown an autosomal mode of transmission and account for cases called familial AD (*f*AD). Mutation of three genes is known to be associated with early-onset familial AD. The mutations in APP gene are the first genetic cause of AD. The mutations are located before β-secretase cleavage site, after α-secretase site, or immediately after γ-secretase cleavage site. In 1995, researchers identified genetic mutations within presenilin 1 gene (PSEN1, chromosome 14) and presenilin 2 gene (PSEN2, chromosome 1) in several early-onset *f*AD (Rogaev et al 1995), these two genes encodes for presenilin 1 and presenilin 2 transmembrane proteins, respectively. Both presenilins are expressed in the brain and many tissues of the human body. It was shown that both proteins are subunits of γ-secretase, which cleaves APP within its transmembrane domain and γ-secretase generates a spectrum of peptides which accumulates in the brain of AD patients.

1.1.3.3 INVESTIGATED GENES IN ALZHEIMER'S DISEASE

AMYLOID PRECURSOR PROTEIN (APP)

APP is a single transmembrane domain protein with multiple alternative transcripts and expressed ubiquitously (Kang et al 1987); has a short half life and is metabolized rapidly by two different pathways in all cells. APP is sensitive to proteolysis by a set of proteases called α, β and γ secretases. Secretases are responsible for the production of Aβ (1-40) peptide or the Aβ (1-42) variant with a significantly higher capacity to self-aggregate. A total of five mutations have been described in the APP gene that lead "purely" to AD. Those that cause the "pure" AD phenotype have in common the fact

that they lead to the production of long Aβ. The phosphorylation of APP on the Thr668-Pro motif has been shown to be increased in AD brain or in models of AD, leading to increased production of Aβ peptides (Lee et al 2003, Pastorino et al 2006). The production of long Aβ is of particular importance because long Aβ has been shown to be the earliest and most abundant specie of Aβ in neuritic plaques, and in biophysical experiments it has been shown to be the most prone to form fibrillar aggregates (Jarret et al 1993).

PRESENILIN 1 (PSEN1)

Several studies indicate that PSEN1 and Presenilin 2 (PSEN2) are predominantly expressed in neurons; however, expression in glia has also been observed (Lah et al 1997). Intracellular localization of the PSEN1 and PSEN2 proteins reveals that they are present predominantly in the endoplasmic reticulum with some immunoreactivity observed in the early Golgi (Kovacs et al 1996). In neurons, both proteins are principally observed in the cell body and in the dendrites (Cook et al 1996). The vast majority of mutations (>80%) in PSEN1 and PSEN2 are associated with early-onset AD. Mutations in both PSEN1 and PSEN2 are associated with increased production of Aβ42 (Mann et al 1997), the amyloidogenic form of Aβ that is deposited selectively in early AD (Iwatsubo et al 1994). PSEN1 is involved in normal APP procesing, therefore, mutations leading to PSEN1 with different altered sites to appear to be responsible for the erroneous cleavage of APP and generation of Aβ 1-42, the most aggressive variant for the plaque deposition in the human brain. Furthermore, PSEN1 interacts with glycogen synthase kinase, one of the critical protein kinases involved in *tau* phosphorylation.

PIN1

PIN1 (peptidyl-prolyl *cis/trans* isomerase) belongs to the parvulin family; it is conserved from yeast to humans and has been shown to be necessary to undergo mitosis (Shen et al 1998). The implications of PIN1 in AD pathogenesis have been reported from several laboratories (Liou et al 2003). The recent finding that PIN1 is oxidatively modified, and that it also shows reduced activity and decreased expression in

hippocampus from MCI and AD subjects (Butterfield et al 2006). Previous studies have shown that PIN1 is co-localized with phosphorylated *tau* and exists in inverse relationship to the expression of *tau* in AD. In addition, an *in vitro* study has shown that PIN1 protects neurons against age-related neurodegeneration (Liou et al 2003); it can also restore the ability of phosphorylated *tau* to bind microtubules and promote their assembly *in vitro*, a process that might represent a potential therapeutic use for PIN1 (Thorpe et al 2001).

PIN1 is mainly localized to neuronal nuclei in normal human brain but in AD brain, it is found in neuronal cytoplasm and perikaryan NFTs. PIN1 in AD brain binds to the phosphorylated Thr231 residue of *tau* protein. The neurodegeneration and neuronal apoptosis in AD might possibly be induced via depletion of nuclear PIN1 or association with specific up-regulated phosphoprotein targets (Thorpe et al 2004).

APOLIPOPROTEIN E

Apolipoprotein E (APOE) exists mainly as a component of lipoprotein complexes along with other apolipoproteins and proteins in plasma and CSF. In humans, there are three major polymorphic forms of APOE: APOE2 (Cys112, Cys158), APOE3 (Cys112, Arg158), and APOE4 (Arg112, Arg158) (Nickerson et al 2000). Several susceptibility genes have also been implicated in AD risk, one of which, APOE, has been confirmed to confer risk for sporadic, late-onset disease (age >60 years), and has also been linked to autosomal dominant *f*AD. The APOE ε3 allele is present in 50–90% of people in all populations, whereas APOE ε4 is present in 5–35% and *APOE* ε2 in 1–5% of people. Risk of AD is associated more strongly with APOE ε4 than it is with ε3 and in turn more strongly with ε3 than ε2. The APOE ε4 allele is present in about 50% of patients who have late-onset disease, compared with 20–25% of controls. Presence of one copy of the APOE ε4 allele increases risk of late-onset AD about three times and two copies about 12 times. Although the mechanism by which APOE isoforms affect risk of AD is not entirely understood, there is strong evidence that APOE isoforms differentially modulate Aβ metabolism and accumulation. *In vitro* studies and studies in animals show that APOE has an important role in determining whether and when Aβ converts from a monomeric, non-toxic molecule into higher-molecular-weight forms such as oligomers and fibrils (Wisniewski et al 1994). In post-mortem tissue from patients with AD, Aβ

plaques are a major hallmark of AD pathology, and APOE is found within these plaques. Several studies have reported an increase in senile and neuritic plaques in patients with AD who were homozygous for APOE ε4 compared with those who were homozygous for APOE ε3 or who had the APOE ε4/ε3 genotype; moreover, more plaques were noted in patients with APOE ε4 than in those without the allele. (Strittmatter et al 1993, Polvikoski et al 1995).

SIRTUIN 1 (SIRT1)

Sirtuin1 (SIRT1) is the mammalian homologue of yeast silent information regulator (Sir)-2, a member of the sirtuin family of protein deacetylases. Among the non-histone cellular substrates of SIRT1 there are the tumor suppressor p53, the transcription factor NF-κB and the FOXO family of transcription factors. SIRT1 also regulates a wide range of metabolic activities in muscle, adipose tissues and liver; therefore have apparent functions that could link nutrient availability and energy metabolism to adaptive changes in transcriptional profiles that affects cell survival in multiple systems. In mouse embryos, SIRT1 was expressed at high levels in the heart, brain, spinal cord, and dorsal root ganglia (Sakamoto et al 2004). The high levels in the brain suggest that might have a role in neuronal and/or brain development. In the adult brain, can be found in the hippocampus, cerebellum and the cerebral cortex, and has been hypothesize that oxidative stress and energy homeostasis can affect the levels of SIRT1 (Wu et al 2006). The sirtuin deacetylation reaction, as seen in neurodegenerative conditions associated with memory impairment, and has been shown to consume NAD^+ (know to protect neurons), causing a release in nicotinamide and ADP, which in turn inhibits sirtuins, this recycling is crucial for the maintaining of SIRT1 functions (Bordone and Guarente 2005) A link between SIRT1 and AD is increasingly evident (Anekonda 2006). Overexpression of SIRT1 and resveratrol treatment markedly reduced NF-κB signaling stimulated by Aβ and had strong neuroprotective effects. Another possible link between SIRT1 and AD came from the potential benefits of caloric restriction on AD symptoms and progression. Recent epidemiological evidence suggest that individuals who maintain a low caloric diet have a reduce risk of developing AD (Mattson MP 2003). Has been propose that SIRT1 induces non-amyloidogenic APP procesing be corroborated by our finding that NAD^+ or resveratrol treatment led to dose-dependent increases in the content of soluble APPα (Bordone and Guarente 2005).

1.1.3.4 MONOZYGOTIC TWINS

Human monozygotic (MZ) twins account for 1 in 250 live births. The origin of MZ twins is attributed to two or more daughter cells of a single zygote undergoing independent mitotic divisions, leading to independent development and births. They are considered genetically identical, but significant phenotypic discordance between them may exist. MZ twins have been used to demostrate the role of enviromental factors in determining complex diseases and phenotypes, but the true nature of the phenotype discordance remains poorly understood. Epigenetics profiles may represent the link between an environmental factor and phenotypic difference in MZ twins.

The study of epigenetic profiles in twins offers an excellent opportunity to understand the causes and consequences of epigenetic variation. Twin epigenetic heritability estimates tell us about the genetic control of DNA methylation variability and the stability of methylation patterns during cell division. The contribution of epigenetic variants to complex phenotypes can be assessed using disease-discordant MZ twins who are otherwise matched for genetics, age, sex, cohort effects, maternal effects and a common environment. Phenotype differences between MZ twins reared apart are not significantly higher that between MZ twin reared together (Hanson et al 1991). Rates of disease discordance in MZ twins are usually well over 50%, even for highly heritable disease (Kendler and Prescott 1999), suggesting that epigenetics can contribute significantly to MZ twin discordance (Petronis 2010). Over the past two decades the discordant MZ twins design has emerged as a powerful tool for detecting phenotype risk factors while controlling for unknown confounders.

1.1.3.5 EPIGENETICS

GENERAL CONSIDERATIONS

Epigenetics (from the Greek, epi-: *επί-* over, above; and -genetics) correspond to the study of heritable changes in gene expression or in the cellular phenotype caused by mechanisms other than changes in the DNA sequence (Feinberg and Tycko 2009). Cellular differentiation is a well know example of relevance of epigenetic mechanism. If all cells within an organism have the same DNA (Nestler 2009) then the ability to have different cells with different functions must be due to a selective activation or silencing of particular genes within genome (Grewal 2003). Actually, it has been demonstrated that epigenetic events, altogether with genetic events, plays a crucial role in tumor progression (Jordà and Peinado 2010).

Three epigenetic mechanisms are considered the most important ones: genomic imprinting, histone modifications and DNA Methylation (Feinberg and Tycko 2004) (Fig 7). Genomic Imprinting refers to the relative silencing of one parental allele compared with the other parental allele as consequence of differentially methylated regions within or near imprinted genes. Histone modifications, principally acetylation, methylation and phosphorylation, are important in transcriptional regulation due the ability to induce chromatin structure modification, altering DNA accessibility (Feinberg and Tycko 2004). DNA methylation is the most common epigenetic mechanism (Jordà and Peinado 2010) and consists in a covalent modification of DNA, in which a methyl group is transferred from S-adenosylmethionine to the C-5 position of cytosine by a family of cytosine (DNA-5)-methyltransferases (Feinberg and Tycko 2004) and occurs predominantly in the cytosines that precede guanines (CpG) (Bird 1986).

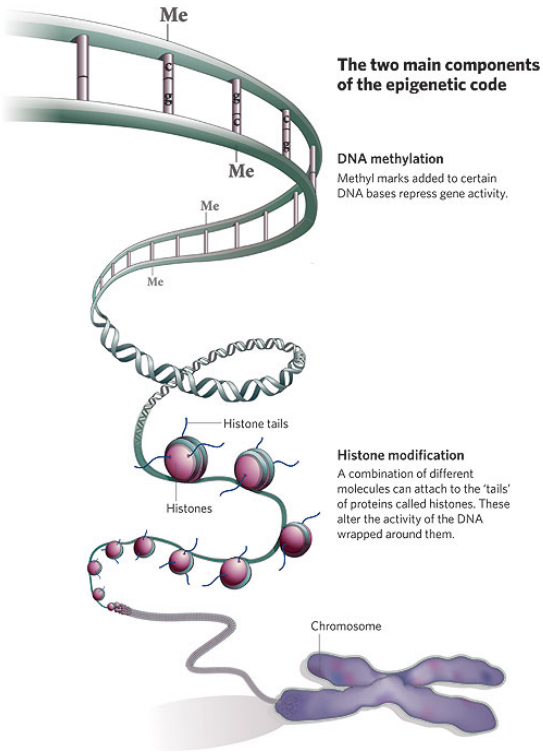


Fig 7.- Esqueme of principal components of epigenetic code, DNA Methylation and Histone modification.

DNA METHYLATION

DNA methylation appears to be one of the most important epigenetic mechanisms used by the cell, for the establishment and manteinance of the correct patterns of gene expresion. Indeed, alterations in the patterns of genomic methylation are strongly associated with several human diseases, making the use of specific inhibitors of the processes involved a common practise in their treatment (Egger et al 2004). DNA methylation patterns are stablished during differentiation, and serve to suppress genes unnecessary for the function of the mature cell. Demethylation of DNA also occurs and involves at least two mechanisms: the first is the mechanism by which 5-azacytidine (an irreversible DNA Mtase inhibitor) hypomethylates DNA, and a second mechanism is that may involve DNA demethylase (Richardson 2003). DNA methylation in mammals ocurrs in the cytosin of the CpG dinucleotide via a reaction catalysed by enzymes named DNA methyltransferases (DNMTs) and the recognition of methylated cytosines is done by proteins that posses a specific binding domain, the so-called methyl-CpG

binding domain. DNMTs are expressed throughout neural development, and in the adult brain in selective regional and cell-specific patterns including mature stem cell generative zones mediating ongoing neurogenesis (Feng et al 2007). Moreover, DNMTs are actively regulated by physiological and pathological states and interactions, and they promote neuronal survival, plasticity and stress responses (Ooi et al 2007). In mammals, the DNMT family includes five proteins: DNMT1, DNMT2, DNMT3, DNMT3B, and DNMT3L (DNMT3-like). On the other hand, the proteins that binds to and recognises 5-methylcytosines are the methyl-CpG binding domain proteins in mammals are MeCP2, MBD1, MBD2, MBD3 and MBD4. MeCP2 was the first of these proteins to be characterised.

The methylation of CG sequences can affect nearby gene expression. Hypomethylation of regulatory sequences ussually correlates with gen expression, while methylation results in transcriptional suppressor. In general, the more CpG islands located in the promoter of a gene, the more the trancription level is dependent on DNA methylation (Graff and Mansuy 2008). Methylation of CpG units disrupts the binding of transcription factors and attracts proteins known as methyl-CpG binding domain proteins that are associated with gene silencing and chromatin compaction (Antequera and Bird 1993). The CpG islands, regions with more than 500 bp and a G + C content larger than 55%, are localized in the promoter regions of 40% of all the genes in mammals and are normaly maintaind in the non-methylated form (Bird and Wolffe 1999, Takai and Jones 2002), but the CpGs located outside the CpG islands are ussually methylated (Urdinguio et al 2009). The importance of DNA methylation in the function of normal cells is evidenced by its role in differentiation, X chromosome inactivation, genomic imprinting maintenance of chromatine structure, and suppression of "parasitic" DNA. Methylated citosines can serve as binding platform for specific proteins. On the other hand, this modification can also prevent binding of proteins to DNA.

It has been observed that multiple exogenous agents can affect DNA methylation, and it is possible that transient exposure to a DNA methylation inhibitor can have long term effects on DNA methylation. Dietary deficiencies in nutrients important for transmethylation reactions are one potential cause of DNA hypomethylation, for example diets deficient in folate, choline and methionine, or trace elements such zinc or selenium (Cooney 2001, Poirier 2002). In AD a diet deficient in folate may, by

increasing homocysteine levels, promote the degeneration of neurons (Kruman et al 2002).

HISTONE MODIFICATIONS

Gene expression can also be modulated by the chromatin state. DNA is packed in the nucleus of eukaryotic cells through its chromatin organisation. The nucleosome, the fundamental unit of chromatin structure, consists of 146 base pairs of DNA wraped around an octamer of histone made up of two copies of each of the core histone (H2A, H2B, H3 and H4) (Kouzarides T 2007). Each core histone is composed of a structured domain and an unstructured amino-terminal tail of varying lengths from 16 amino acid residues for H2A, 32 for H2B, 44 for H3 and 26 for H4, protruding outward from the nucleosome (Taniura et al 2007). These proteins provide not only a solid structure; N-terminal regions of histones which protrude from the nucleosome are susceptible to interactions with other proteins. Chromatin can exist either in a decondensated, active arrangement, termed euchromatin, or in a condensated, inactive state, i.e. heterochromatin.

The post-translational modification of the residues at histone tails are: methylation of lysines and arginines, acetylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation. Two widely studied histone modifications are histone acetylation and phosphorylation. Histone acetylation is linked with transcriptional activation, while deacetylation is related to transcriptional repression (Berger 2007). Histone acetylation is a reversible modification of lysine residues within the amino-terminal tail domain of core histone; histone acetyltransferase (HATs) transfers an acetyl-group from acetyl-coenzyme A to the ε-amino group of the lysine resiue, while histone deacetylase (HDACs) acts in the reverse to remove the acetyl group. Also histone can be methylated by histone methyltransferases, inducing changes in the chromatin structure. Methylation may create binding sites for other regulatory proteins thus influencing the chromatin structure, either condensating or relaxing the structure (Chouliaras et al 2010).

Although DNA methylation and histone modifications can act independently, they can also interact with each other. DNA methylation is associated with histone modifications through methyl CpG binding proteins interaction with dynamic complexes containing

histone-modifying enzymes that promote gene repression and DNA replication and repair (Klose and Bird 2006). The binding of some deoxymethylcytosine binding proteins to methylated sequences attracts complexes containing co-repressors and histone deacetylases, leading to a change in the chromatin structure from an open, trancriptionally active form to a more compact, inactive form, inaccessible to the transcription machinery (Richardson 2003).

EPIGENETICS AND ALZHEIMER'S DISEASE

Although the putative role of epigenetic mechanisms in the pathophysiology of AD has not been yet investigated, several characteristics of late-onset sporadic AD are compatible with an epigenetic component (Chouliaras et al 2010). Several studies in MZ twins have shown that the epigenetic code diplays alterations, for example difference between the genomic 5-methyl-C content and the acetylation levels of H3 and H4 were significantly different in each twin. Also has been found that a different association in this epigenetic difference and the age of the MZ twin, i.e. the younger pair were epigenetically similar, whereas the older pairs were most distinct (Fraga et al 2005). In fact, some of enviromental exposures that have been associated with an increased risk of developing AD have also been shown to induce epigenetic changes in a diversity of tissues samples. In addition, aging which represents one of the major risk factors of AD, is asociated with remarkable epigenetic alterations (Fraga 2009).

Several studies have been conducted on altered DNA methylation in AD. Most of these have analyzed methylation status of the promoters of genes implicated in the pathophysiology of AD in human postmortem brain tissues. There is no clear pattern for methylation in patients with AD. A global DNA hypomethylation was observed in the cortex of AD patient (Mastroeni et al 2008) for the promoter of the gene encoding CREB5, a trancription factor involved in synaptic plasticity and cognition (Zukin 2009) but in particularly, in the promoter region of the APP gene in patient with AD when compared to controls. Also has been showed that CpG island in the promoter region of the APP gene in the parietal cortex are frequently methylated before the age of 70 years and are significantly demethylated after that age, which may be associated with the progressive deposition of Aβ in the aging brain (Tohgi et al 1999). Another studies reported increased methylation in the promoter regions of the APOE and MTHFR genes

in both postmortem prefrontal cortex tissue and peripheral lymphocytes of AD patients were hypermethylated when compared to controls (Wang et al 2008). The finding that lymphocyes in people with sporadic AD also showed an age-specific epigenetic drift, although the differences were smaller than in brain tissues, suggest that epigenetic changes may affect also peripheral cell types (Chouliaras et al 2010). While that the expression patterns of lymphocytes may differ substantilly from the expression pattern in the CNS findings that gene expression profiling of lymphocytes in AD patients and healthy aged controls showed a difference in the expression of 20 genes involved various pathways, suggest that changes in accessible peripheral cell types could be useful as biomarkers for AD (Kálmán et al 2005).

Regarding the histone modifications has been reported that the examination of brains of MZ twins discordant for AD, the pathology of the disease was associated with a marked increased of the trimethylation of histone H3K9 and condensation of heterochromatin structure in the temporal cortex and hippocampus of the twin with AD when compared to the other twin. H3K9 trimethylation is a marker of gene silencing. Otherwise it have been observed that the administration of HDAC inhibitors, like sodium butyrate, reinstated learning behavior and contributed to the recovery of long-term memories in the mouse model of neurodegeneration (CK-p25 TG) (Sweatt 2007). In line with a central role for HDACs, overexpression of HDAC2 in mice was shown to induce significant memory impairment (Guan et al 2009).

It is evident that AD is associated with epigenetic dysregulation at variuos levels and that epigenetic mechanisms may mediate the effects of life events on AD risk. Indirect evidence demostrating epigenetic alterations associated with various risk factors for AD, such as nutritional factors, stress, depression, and brain trauma, implies that epigenetic processes may be the key mechanism mediating genotype and enviromental factors interaction in AD.

1.1.3.6 PERIPHERAL BLOOD MONONUCLEAR CELLS

Unlike many diseases, in which researchers and doctors are able to access the affected tissue, while the patient is still alive, psychiatry is limited by inaccessibility of the organ of interest. Therefore, there exists a long history of research of peripheral markers capable of reflecting the pathology within the brain. There are several factors that make it particularly useful the Peripheral Blood Mononuclear Cells (PBMC) to serve as a model of epigenetic gene regulation in the brain.

First, previous studies have shown that PBMCs may provide a reliable means to study the impact of environment / life experiences on the structure of chromatin and DNA methylation. Fraga et al 2005 has reviewed a number of epigenetic parameters of lymphocytes in MZ twins aged between 3 and 70 years and found that while 3 years twins were virtually indistinguishable in terms of their overall level of DNA methylation, acetylated histone 3 and 4, 50 years twins have had significant differences on these measures. In general, elderly twins were more different in terms of epigenetic parameters than younger twins. Moreover, it is important to note that these differences were consistent across subjects in at least 12 weeks, indicating that measures of global epigenetic parameters in peripheral lymphocytes are a reliable method to assess the state of chromatin. These results indicate that chromatin extracted from lymphocytes may provide a "molecular fingerprint" reflecting the environment and life experience of an individual, and stochastic factors that would not have been revealed through other tests. Secondly, the analysis of gene regulation in nucleated blood cells of live patients takes fully account of the evolution of the disease, including drug response, metabolic and environmental events, and is the only approach likely prospects for the longitudinal clinical research, and seems to be the natural evolution of post-mortem studies on the brain. Additionally, PBMCs contain the complete set of enzymes and epigenetic mechanisms founded in many tissues including neurons (De Ruijter et al 2003, Dangond and Gullans 1998). Previous studies have shown that PBMCs are able to exhibit all the anomalies of the epigenetic mechanisms, also probably present in the brain. For example, in HD, a disease known to be associated with histone acetyltransferase malfunctioning, has been found a similar pattern of transcriptional repression in various chromosomes in both blood and brain (Anderson et al 2008). In addition, several studies have shown that peripheral markers are able to discriminates

the differences of chromatin structure in twins discordant for mental illness (Kuratomi et al 2008, Tsujita et al 1998), as well as show similarities in epigenetic parameters between individuals with the same disease (Kuratomi et al 2008, Petronis et al 2003).

It is possible to hypothesize that PBMCs may be able to reflect epigenetic mechanisms within an individual and provide a mean to discriminate such subsets of patients who have profound abnormalities of chromatin structure or DNA methylation. This approach could also help to understand the impact of hormones, drugs and drugs of abuse on chromatin. Finally, it could provide a tool that helps in the development of new chromatin altering agents as well as to identify patients most likely to benefit from these types of drugs.

2. AIM OF THE STUDY

During the last decade, the molecular studies of neurodegenerative disease have had a significant increase, due to their multifactorial etiologies. From environmental to genetic factors seem to be involved in their pathogenesis. It's well accepted that many of the underlying pathogenic processes are similar for several neurodegenerative diseases, and includes protein misfolding, oxidative stress, cytoskeletal abnormalities, disruption of calcium homeostasis, and inflammation, all of which increase during aging. Thus, aging is described as the most significant risk factor of neurodegenerative disorders. During aging process several disturbances leads to DNA damage, suggesting an association between age and epigenetic changes. The existence of related mechanisms underlying neurodegeneration raises the possibility of developing a class of therapeutic strategies usefull for a variety of neurological disorders through activation of body's own defenses against age-related changes that leads to deterioration and cell death. Molecular and epigenetics mechanisms offer the possibility to develope new indicators or "markers" to improve early detection and diagnosis rate. For this reason the research was focalized in the two most important neurodegenerative diseases: Parkinson's and Alzheimer's. Their have a strong genetic component, but the sporadic presentation is around 90% of cases.

Thus, the first part of this thesis concerns to the study of molecular mechanisms of PD using an animal model. It has been described that using animal models allows to replicates the pathology and symptomatology and is essencial for the development of new therapies. We developed an animal model based on two pestices commonly used in agriculture, PQ and MB. The dopaminergic damage was confermed principally by TH and SNCA levels, and the end we evaluated the toxic effects in the transcription of NOFQ/NOP system genes in dopaminergic neurons of rats.

On the other hand, as already mentioned, it has become increasingly obvious that epigenetic mechanisms are an integral part of a large number of brain functions that range from the development of the nervous system and basic neuronal functions to higher order cognitive processes. For this reason, the second part of this thesis was directed to evaluate peripheral markers in PBMC samples of MZ twin discordant for

AD. Specifically, we evaluated the relation between epigenetic mechanisms and the transcription of genes involved in AD, using the two most importante epigenetics codes, DNA methylation and histone modification. We determinated the gene expression and methylation levels of APP, PSEN1, SIRT1, PIN1 and APOE promoter region, in MZ twins discordant for AD; as well as the immunoreactivity of three histone modifications, H3K9 Ac, H3K4 me3 and H3K27 me3.

3. MATERIALS AND METHODS

3.1 PARKINSON'S DISEASE

3.1.1 CHEMICALS

Paraquat dichloride (1,1′-dimethyl-4,4′-bipyridinium dichloridehydrate) and Maneb (manganese-ethylenebisdithiocarbamate) were purchased from Sigma (Milan, Italy).

3.1.2 EXPERIMENTAL ANIMALS AND TREATMENT

Adult male Sprague–Dawley rats, weighing 170 ± 10g at the beginning of treatment, were used (Harlan, Correzzana, Italy). Rats were housed three per cage in a temperature (22 ± 1 °C) and light (8:00 a.m.– 8:00 p.m.) controlled room for at least 6 days before use. Standard rat chow and tap water were available *ad libitum*. Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and national laws and policies (Ministry of Health authorizations no: 204/2008-B).

Rats were injected intraperitoneally (i.p.) twice a week (on monday and thursday) for 4 weeks (total of 8 injections), with saline solution (vehicle, $n = 10$), PQ + MB (10 + 30 mg/kg, $n = 10$) referred as high dose (HD) and PQ + MB (5 + 15 mg/kg, $n = 10$) referred as low dose (LD). Doses have been chosen based on previous reports (Thiruchelvam et al 2000, Cicchetti et al 2005). Pesticides were dissolved in saline solution and the combinations were administrated as separated injections.

3.1.3 WEIGHT, LOCOMOTOR ACTIVITY AND BEHAVIOUR

Animal weights were registered every morning before injection. Locomotor activity was measurement with Actometric cages (38 x 30 x 25cm). A DC current, 65 V, 25 μA was continuously delivered to the stainless steel grid floor of the cage and every closure of the circuit performed by the rats feet was recorded as one motility count by an electronic counter, only horizontal displacements of the animal across the cage were

recorded. Animals have one hour habituation period to the experimental environment. At the end of this time, each 10 minute during one hour the horizontal and ambulatory movements were recorded. Behavioural assessment was performed by direct observation 24 hours after injection by the same observer during all treatment. The parkinsonism following PQ and MB exposure were rated, for a maximal disability score of 9 in the following way: (a) Posture: normal = o, flexed intermittent = 1, flexed constant = 2, crouched = 3; (b) Mobility: normal = 0, mild reduction = 1, moderate reduction = 2, severe reduction = 3; (c) Gait: normal = 0, slow = 1, very slow = 2, very slow with freezing = 3. A score was given every 30 min reflecting observations of the preceding half-hour (modify of Hadj Tahar et al 2001).

3.1.4 TISSUE PROCESSING

Seven days after end of treatment, rats were killed by decapitation and their brains were removed. The SN and CP were rapidly dissected out, frozen on dry ice and stored at -80° C until use.

3.1.5 WESTERN BLOTTING

Protein Extraction

The tissue was sonicated in 4 volume of lysis buffer (50 mM Tris, pH 7,5, 0,4 % NP-40, 10% glycerol, 150 mM NaCl, 10 g/ml leupeptin, 10 mM EDTA, 1 mM sodium orthovanadate, 100 mM sodium fluoride) and centrifugated at 12000 rpm at 4°C for 20 minute, the supernatant was stored at -20°C.

Quantification Protein

The protein concentration was determinated by the Bradford protein assay (Bio-Rad Laboratories, Inc. Milan, Italy).

Western Blotting Analysis

Close to 30 μg of protein were separated by 10% SDS-polyacrilamide gel and then transfered by electrophoresis for 3 h at room temperature to a 0.45 μm nitrocellulose membrane (Bio-Rad, Italy). The membrane was blocked for 60 minute with a mixture of 5% non-fat dry milk and TBS-T 1%, and incubated overnight at 4°C with TH,

SNCA, P-ERK 1/2, ERK 1/2, P-CREB, CREB, GAD 65/67 antibodies at different concentration with the appropriated secondary antibodie conjugated with horseradish peroxidase (Table 1). Then, membranes were washed five times with TBS-T 1x and incubated for 1 h at room temperature with a horseradish peroxidase-linked anti-rabbit secondary antibody and horseradish peroxidase-linked anti-mouse secondary antibody when appropriated (Table 1). The immunoreactivity was visualized by enhanced chemiluminiscence (ECL) detection solutions in to Versadoc MP 4000 (Bio-Rad). The membrane was reprobed with a mouse anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH) monoclonal antibody, and with a horseradish peroxidase-linked anti-mouse secondary antibody (Table 1). For quantitative analysis of obtained bands, Quantity One software (Bio-Rad) was used. The integrated intensity of the band (optical density value) was determined using both band density and area. The variations of proteins levels were then evaluated with respect to GAPDH. The resulting ratios were evaluated as level percentage change or as changes in status of activation, expressed as the ratio between the phosphoprotein and total protein compared to controls, with controls as 100%. The results were then reported as histograms.

PRIMARY ANTIBODIES	KDa	DILUTION
Antibody policlonal anti-Tyrosin Hydroxylase	55	1:1000
Antibody policlonal anti GAD $_{65/67}$	65/67	1:2000
Antibody policlonal anti-CREB	43	1:1500
Antibody policlonal anti-CREB phosphorylated Ser 33	43	1:400
Antibody anti-ERK ½	42/44	1:2500
Antibody anti-ERK ½ phosphorylated	42/44	1:2000
Antibody alpha-synuclein	25	1:500
Antibody monoclonal anti-GAPDH	36	1:3000

SECONDARY ANTIBODIES	DILUTION
Antibody anti-rabbit horse radish peroxidase	1:3000
Antibody anti-mouse horse radish peroxidase	1:3000

Table 1. Proteins and antibodies concentrations used for Western Blotting analysis.

3.1.6 REAL TIME RT-PCR

Extraction and measurement of total RNA.

Total RNA was prepared according to the method previously described (Chomczynski and Sacchi 1987). The RNA was extracted from single tissue samples by homogenizing with TRI Reagent solution (Ambion Inc. Italy), containing phenol and guanidine thiocyanate (Ambion), 1 mL TRI Reagent solution per 50-100 mg tissue. Then, 0.2 ml chloroform/2 ml of homogenate, and centrifuging the suspension at 12,000 x g for 10-15 minute at 4°C, and was transfered the aqueous phase to a fresh tube. A volume of 0.5 ml isopropanol was added, incubated for 15 min at 4°C and the RNA pellet was isolated by centrifugation at 12,000 x g for 25 min at 4°C (Di Benedetto et al 2009). The pellet was washed twice with 75% ethanol, dried under vacuum and then resuspended in 25 µl of Rnase-free water. Total RNA, digested with DNase RNase-free enzyme to eliminate genomic DNA content, was quantified by measurement of absorbance at 260 nm (1OD/ml = 40 µg RNA/ml). The ratio OD260/OD280 > 2 provided an estimate of the purity of the total RNA.

Real Time RT-PCR analysis

RNA samples were subjected to DNase treatment and converted to cDNA with the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) by using random hexamers (0.45 µg of total RNA in a final reaction volume of 20 µl). The cDNAs were subsequently diluted three times. Relative abundance of each mRNA species was assessed by real-time RT-PCR employing 1 µl of the diluted samples in a final volume of 20 µl using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on an DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined and the point of early log phase of product accumulation is defined by assigning a fluorescence threshold above background defined as the threshold cycle number or Ct. Differences in threshold cycle number were used to quantify the relative amount of PCR target contained within each tube. Relative expression of different gene transcripts was calculated by the Delta-Delta Ct (DDCt) method and converted to relative expression ratio (2-DDCt) for statistical analysis (Pfaffl 2001, Livak and Schmittgen 2001). All data were normalized to the endogenous reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Results on RNA from PQ and MB treated animals were normalized to results obtained on RNA from the control, vehicle treated rats. After PCR, a dissociation curve (melting curve) was constructed in the range of 60 ºC to 95 ºC to evaluate the specificity of the amplification products. The primers used for PCR amplification (Table 2) were designed using Primer 3. Total RNA was converted to complemetary DNA (cDNA) using 50 U Superscript II Reverse Transcriptase (Invitrogen, Milan, Italy) in 20 μL of buffer containing 0.5 mM deoxynucleotide triphosphates (Invitrogen, Milan, Italy), 40 U RNase inhibitor (Invitrogen, Milan, Italy) and 0.5 μg Oligo (dT) 12-18 Primer (Invitrogen, Milan, Italy).

PRIMERS	Forward (5'-3')	Reverse (5'-3')
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT
PARKIN	ACACCCAACCTCAGACAAGG	GACCAAGACAGGGTTCCTGA
KOP	TTGGCTACTGGCATCATCTG	ACACTCTTCAAGCGCAGGAT
NOP	AGCTTCTGAAGAGGCTGTGT	GACCTCCCAGTATGGAGCAG
pDYN	CCTGTCCTTGTGTTCCCTGT	AGAGGCAGTCAGGGTGAGAA
ppN/OFQ	TGCAGCACCTGAAGAGAATG	CAACTTCCGGGCTGACTTC

Table 2.-Sequence of primers used for gene expression of GAPDH, Parkin, KOP, NOP, PDYN and ppN/OFQ.

3.1.7 STATISTICAL ANALYSIS

The data were analyzed by One-way ANOVA, followed by a post-hoc Dunnett t-test. Stadistical significant was set at *P < 0.05 **P < 0.01 versus control group. Results are reported as mean ± SEM for groups.

3.2 ALZHEIMER'S DISEASE

3.2.1 REAL TIME RT-PCR

PBMCs were separated by density gradient (Lympholyte-H, Cedarlane, Canada), total RNA was isolated as reported (Chomczynski and Sacchi 2006) and reverse-transcribed using the M-MLV Reverse-Transcriptase System and oligo (dT) (Clontech, Italy).
Relative abundance of each mRNA species was assessed by real-time RT-PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), on an DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA), and was calculated by the Delta-Delta Ct (DDCt) method, using GAPDH as internal control. The primers used for PCR amplification are indicating in Table 3.

PRIMERS	Forward (5' – 3')	Reverse (3' – 5')
PIN1	GACGAGGAGAAGCTGCCGCC	CAGGCTCCCCCTGCCCGTTT
APP	CATCCCCACTTTGTGATTCC	GTTTCGCAAACATCCATCCT
SIRT1	GCGATTGGGTACCGAGATAA	GTTCGAGGATCTGTGCCAAT
PSEN1	TTGCGGTCCTTAGACAGCTT	AGGACAACGGTGCAGGTAAC
APOE	CCAGCGGAGGTGAAGGAC	TACCTGCCAGGAATGTGA
GAPDH	ATTCCACCCATGGCAAATTC	TGGGATTTCCATTGATGACAAG

Table 3.-Sequence of primers used for gene expression of PIN1, APP, SIRT1, PSEN1, APOE and GAPDH.

3.2.2 DNA METHYLATION

In order to assess the possible methylation status of the promoter region of studied genes in MZ twins discordant for AD, the following approach has been performed:

- DNA extraction from PBMC (Peripheral Blood Mononuclear Cells)
- Bisulfite treatment of extracted DNA
- Analysis of treated DNA by Real-Time PCR

DNA extraction and purification from whole blood

DNA was extracted from a 7 ml blood EDTA sample. Blood samples were frozen (< -20°C) for several days before extraction in order to facilitate leukocyte lysis. FlexiGene (Qiagen) was used to extract genomic DNA following manufacterer's instuctions which main steps are mentioned below.

1. To FG1 Buffer (lysis), contained in 50 ml falcon tube, add 7 ml of whole blood and vortex to mix well.
2. Centrifuge 2500 rpm for 30 min at 10°C. Centrifugation separate proteins (and other macromolecules and sub cellular structures) according they dimensions (and form) through generation of gravitational forces within a sample tube.
3. Surnatant is discarded and FG2 Protease Buffer (denaturation buffer) is added and inmediatly vortexed, the resulting volume is then divided in 1,5 ml eppendorf.
4. Incubate aliquots at 65° for 10 min: color changes from red to green when proteic digestion verifies.
5. Add isopropanol (100%) and mix until DNA precipitation.
6. Centrifuge 10000 rpm for 10 min at room temperature and discard surnatant.
7. Add ethanol 95 % and vortex 5 seconds.
8. Centrifuge 10000 rpm for 10 min at room temperature and discard surnatant.
9. Dry DNA pellet at room temperature until full diluent evaporation (at least 5 min).
10. Add FG3 Buffer (hydratation buffer), vortex 5 seconds at low speed, resuspend the DNA pellet and incubate for 1 hour at 65°C bath.

Sample DNA amount was determined by spectrophotometry at 260 nm and DNA aliquots were frozen at -20°C.

Sodium Bisulfite treatment

The vast majority of DNA methylation analysis is based on using a PCR using DNA treated with sodium bisulphite as a model. Two different strategies are used in the design of the primers for these reactions:

- Methylation-indipendent PCR primers (MIP)
- Methylation-specific PCR primers (MSP)

Normally, the epigenetic information is lost during the PCR because the DNA polymerase does not distinguish between methylated and non-methylated cytosine, so the polymerase adds a guanine and then a non-methylated cytosine in both situations. After PCR, each originally methylated allele is diluted to a concentration impossible to analyze, so the DNA must be modified in a way that allows methylated information to remain preserved. Treatment with sodium bisulfite, which deaminates cytosine into uracil (Clark et al 1994) is the method of choice in most laboratories for this type of analysis.

Due the fact that the degree of deamination of 5'-methylcytosine to thymine is much slower than the conversion of cytosine to uracil, it is assumed that the only remaining cytosine after treatment with sodium bisulfite are those derived from 5'metilcitosine. Thus, during the subsequent PCR, the uracil residues are transcribed as cytosine. The procedure is based on the chemical reaction of single-stranded DNA with sodium bisulfite (HSO3-) at low pH and high temperatures. The chemical reaction of each step is as follows: cytosine carbon-6 sulfonation, irreversible hydrolytic deamination of carbon-4 that produces a sulfonate uracil, and finally the following desulfonation under alkaline conditions to generate uracil. Methylation of carbon-5 prevents the carbon-6 sulfonation in the first step reaction. Although the 5-methylcytosine can react with sodium bisulfite, this reaction is extremely slow, and the balance favors the 5-methylcytosine rather than thymine (the deamination product of 5-methylcytosine). Thus it is important the subsequent necessary purification to remove salts and other reagents used in the process. Treatment with sodium bisulfite converts unmethylated cytosine of the original strand of DNA to uracil, while methylated cytosines remain cytosines.The CpG dinucleotide is the target of methylation in human cells (Fig 8).

The protocol described by Frommer et al 1992 has been widely used for the treatment with sodium bisulfite. When treatment with sodium bisulfite is conducted under appropriate conditions, the expected convertion level of unmethylated cytosines is about 99% (Taylor et al 2007). Despite this high level of conversion, however, it is possible that a small amount of DNA have a lower conversion rate (Warnecke et al 2002) and the

distribution of unconverted sites does not be random, so some promoter regions are more prone to an incomplete conversion. The conversion rate depends greatly on the quality of DNA (Warnecke et al 2002). This is especially important to keep in mind when looking for low levels of DNA methylation with MSP primers based methods.

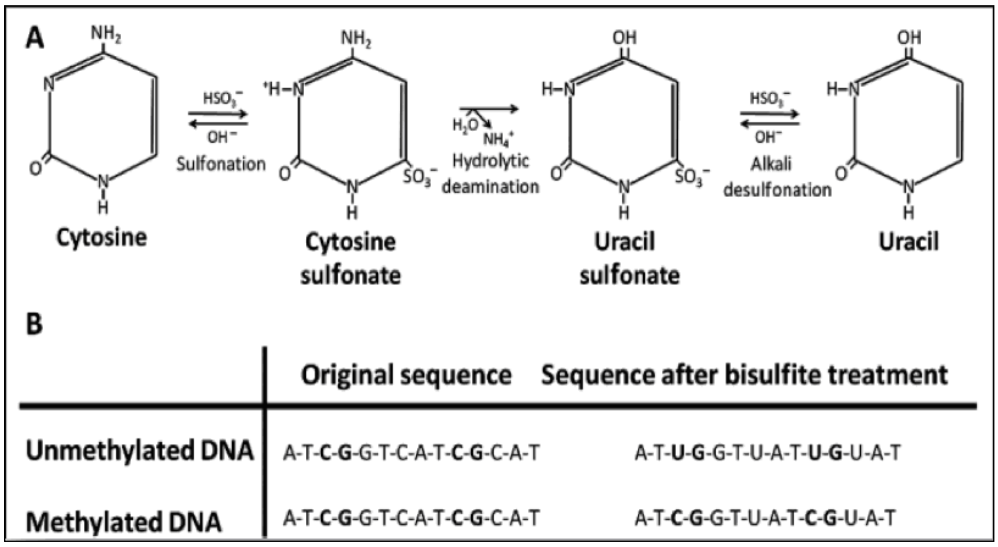


Fig 8.- Sodium Bisulftine treatment of Genomic DNA.

The antiparallel strands of DNA are no longer complementary after treatment with sodium bisulfite. Therefore, the MIP and MSP primers are designed to be both Forward and Reverse.

In mammalian DNA, the major base modification is 5-methylcytosine (5-MC), this occurs in 2-5% of all cytosine residues (generally those that are found in CpG doublets). The modification with bisulfite is a reaction between the molecule bisulfite and unmodified cytosine of single-stranded DNA. The reaction converts cytosine into uracil, while methylated cytosine (5-MC) within the CpG sites remains unchanged. The modified DNA can be amplified via PCR to understand the state of methylation.

The bisulfite modification of DNA samples was carried out with the EpiTect bisulfite kit (Zymo) (Fig. 9) as described by the manufacturer. For each conversion reaction 1 ug of DNA was used. After conversion, the modified DNA was purified and eluted in 20 ul of TE (2,5 mmol / L EDTA, 10 mmol / L Tris-HCl (pH 8)), then used immediately or

stored at -20 ° C for one month. Positive control (100%) and standard curves were produced using universally methylated (uDNA) and methylated (mDNA) DNA.

DNA Input: each treated sample have 500 ng of DNA
Conversion efficiency: >99% of unmethylated Cytosines are converted to Uracil
DNA Recovery: >80%

- Add 5uL of M-Dilution Buffer to DNA sample. Add Nuclease-Free water to a final volume of 50µL.
- Incubate 15 min at 37°C, and add 100µL of CT Conversion Reagent to each sample. Incubate for 12 to 16 hours at 50°C.
- At the end of incubation time, transfer each sample to individual separation columns, containing 400µL of M-Binding Buffer. After the first centrifugation, the DNA remains attached to the resin present in the column while the buffer is discarded.
- Add M-Desulphunation Buffer and incubate 15-20 min.
- Add M-Wash Buffer and centrifuge to wash the DNA.
- Add 20 µL of M-Elution Buffer to release DNA from the resin.

Converted DNA can be conserved at -80° for up to three months.

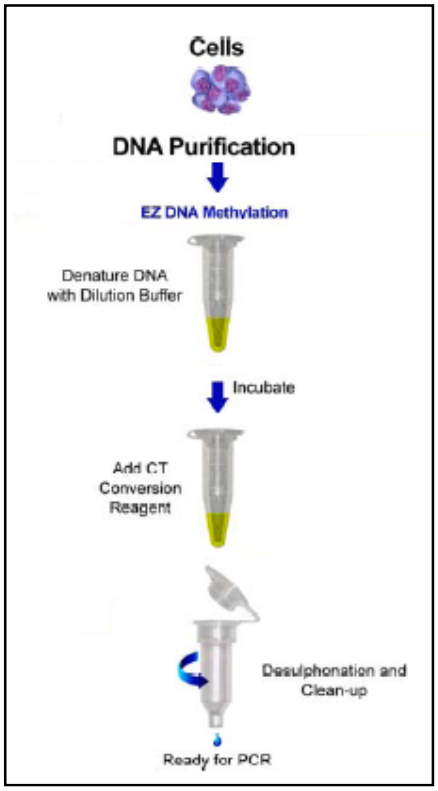


Fig 9.- EZ DNA Methylation procedure (source ZIMO RESEARCH 2010)

MSP primers

The MSP primers are designed to amplify only the methylated DNA thus the possible errors associated with MIP based methods are no longer a problem. This specificity is achieved by including a few CpG sites in the sequence of the primer, preferably at the 3' end (Fig. 10) (see Table 4 for MSP primers).
If PCR strict conditions are followed, only the amplification of methylated DNA will occurs. MSP assays are generally associated with high levels of false positives, especially when using large numbers of PCR cycles, which are often necessary to obtain high-sensitivity analytical assays. False-priming events (in which the amplification takes place despite the mismatch between primer and sample) and not fully converted DNA molecules may be responsible for false-positive results. The events of false-priming can be detected through the use of an appropriate negative control and prevented by limiting the number of cycles and using higher annealing temperatures.

The present study analyzes four genes, APP, SIRT1, APOE, PSEN1 and PIN1. We also used MYOD_noCpG. Of each of these genes we create their MSP primers in order to perform MSP Real-Time PCR.

After retrieving the correct sequence of special databases of the gene of interest, we look upstream in the promoter region for CpG rich areas (CpG Island). Several pairs of suitable sequences are tested (forward / reverse primers).

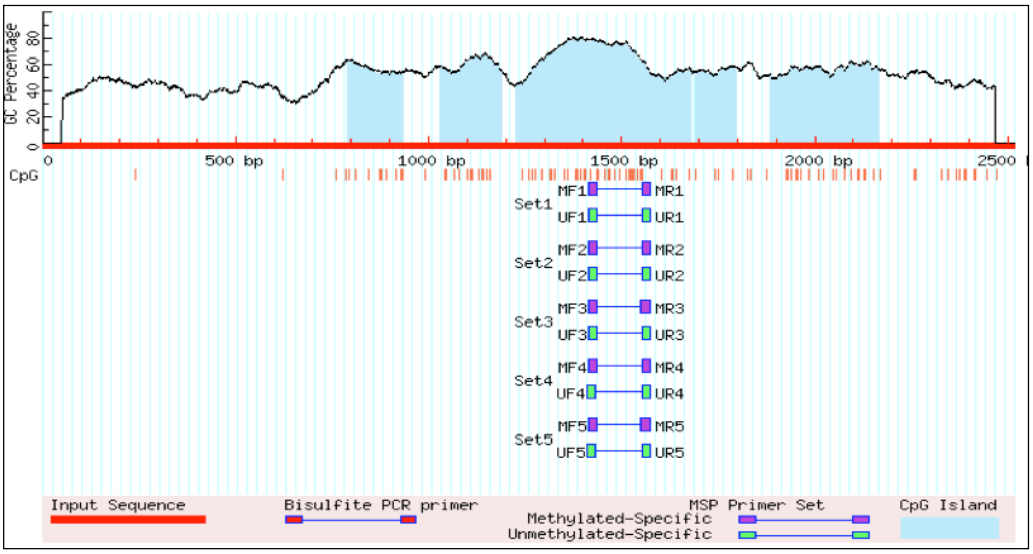


Figura 10.- Graphic representation of CpG rich areas (CpG Island) within gene promoter region.

PRIMERS	Forward (5' – 3')	Reverse (3' – 5')
PIN1	GTCGTTTCGGATTATTTAGGAGTC	TAACTAACCGCGCTCTACACCG
APP	CGTTTGTTTTATTTTTTTAAATCGA	ACGACCCACCTAAACTTCGTA
SIRT1	CGGATTAAAATTTGAGTTGTTTC	CCTTCCTCTTTATAACGAACGTA
PSEN1	GGGGTTTTCGTTTTTAGTTC	AACGATTACGAAAAAAACCC
APOE	ATTTCGGAATTGAGGGGTAC	CTCGAAACGAACCCAAAC
Myod no CpG	CCAACTCCAAATCCCCTCTCTAT	TGATTAATTTAGATTGGGTTTAGAGAAGGA

Table 4.-Sequence of MSP primers used for MSP RT-PCR.

MSP Real-Time PCR

For the methylation PCR study, a SYBR Green PCR kit was used. Each 20 ul of PCR reaction contains: 2 ul of eluted bisulfite modified DNA, 10 ul of SYBR Green PCR Master Mix, 1 ul of each of the two primers (concentrations used vary from 10 to 100 pmol / ul resulting in final concentrations of 0.3 to 3 uM), and 6 ul of DNase-free water. To normalize for the amount of input DNA, we have chosen a pair of primers corresponding to a specific sequence of MyoD gene. Real-time PCR conditions were 95 ° C for 15 minutes followed by 45 cycles of 94 ° C for 15 s, 60 ° C for 30 s, 72 ° C for 30 s with data acquisition after each cycle. At the end, the amplification products will be verified by melting curve analysis: 95 ° C for 1 min, 55 ° C for 1 min, followed by 80 cycles of increasing incubation temperature for 10 s each, from 55 ° C to up to 95 ° C (0.5 °C increase) with data acquisition after each cycle. Two replicates for each sample were used and PCR was performed in a DNA Engine Opticon 2 continuous fluorescence detection system (MJ Research, Waltham, MA, USA). Ct values of each sample were recorded. Methylation percentage was calculated by 2^(-DDCT), where DDCT = (Ct Target - Ct, MyoD) sample - (Ct Target - Ct, MyoD) fully methylated DNA and multiplied by 100. A calibration curve using the fully methylated DNA was carried out in parallel with each analysis, providing additional confirmation for each sample as methylation ratio, defined as the ratio of the fluorescence emission intensity values of target PCR product respect those of Myod PCR products. The correct length and purity of PCR products were verified by agarose gel electrophoresis (1,5% agarose).

TEMPERATURE	APP	SIRT1	APOE	PSEN1	PIN1
Gene expression	60°C	60°C	60°C	60°C	55°C
DNA methylation	55°C	55°C	55°C	55°C	57°C

Table 5.- Annealing temperature for gene expression and DNA methylation of studied genes.

3.2.3 WESTERN BLOTTING

Histone modifications analysis

For protein extraction, PBMCs were lysed in Triton X-114/Tris buffer in the presence of a protease inhibitor cocktail (Sigma, Italy). Extracted proteins (20 μg) were separated

by 15% SDS-polyacrilamide-resolving gel under reducing conditions, and transferred to 0.45 μm nitrocellulose membranes (Bio-Rad, Italy). Bands were immunovisualized with monoclonal antibodies against either Histone 3 lysine 27 trimethylated (H3K27me3) (Millipore, 17-622), Histone 3 lysine 4 trimethylated (H3K4me3) (Abcam, ab8580), Histone 3 lysine 9 acetylated (H3K9Ac) (Millipore, 17-658) or GAPDH, the latter used to normalize the acetylation and methylation status of H3, followed by a horseradish peroxidase-linked anti-mouse antibody and a chemiluminescent substrate (Amersham Biosciences Corp., Milan, Italy) (Table 6). Membranes were scanned and the intensities of the immunoreactive bands were quantified by densitometry, using a molecular analysis software system (Bio-RAD GS-700 Imaging Densitometer).

PRIMARY ANTIBODIES	KDa	DILUTION
Antibody Anti-acetyl-Histone H3 (Lys 9)	17	1:1000
Antibody Anti-trimethyl-Histone H3 (Lys 4)	17	1:5000
Antibody Anti-trimethyl-Histone H3 (Lys 27)	17	1:1500
Antibody monoclonal anti-GAPDH	35	1:3000

SECONDARY ANTIBODIE	DILUTION
Antibody anti-rabbit horse radish peroxidase	1:3000
Antibody anti-mouse horse radish peroxidase	1:3000

Table 6.-Proteins and antibodies concentrations used for Western Blotting analysis.

4. RESULTS

4.1 PARKINSON'S DISEASE

We observe a decrease of weight in all animals after the treatment with the association of PQ + MB HD and LD, being statistically significant versus control group at the same time of injection (Fig 11). The animals treated with PQ + MB HD and LD (Fig 12) showed a significant decrease of locomotion (204 ± 28 steps and 295 ± 52 steps of control, respectively). Regarding the results of behavior both associations revealed an increased statistically significant in the levels of disagreement of the animals (Fig 13).

The immunoreactivity observed in western blotting revealed decreased levels of TH in the SN after the treatment with the association of PQ + MB HD and LD (78,4 ± 10,1 % and 39,7 ± 14,5 % of control, respectively), being statistically significant only for PQ + MB LD. We observed a decrease in TH immunoreactivity in CP after PQ + MB HD and LD (85,3 ± 8 % and 65,9 ± 5,5 % respectively compared with Control) (Fig 14), with statistically significant differences only after the association of PQ + MB LD. The levels of SNCA, assessed in the western blotting analysis, showed an increase in the PQ + MB HD and LD groups in the SN (132,1 ± 11,1 % and 153 ± 13 % of control, respectively) and CP (162,6 ± 13,8 % and 130,9 ± 14,9 % of control, respectively), being statistically significant in both areas (Fig 15). The levels of Parkin mRNA revealed a significant decreased in the SN after the treatment with PQ + MB HD and LD (57 ± 9 % and 67 ± 5 % of control, respectively), no significant modifications was observed in the CP (98 ± 9 and 92 ± 17 % of control, respectively) after both treatment (Fig 16).

P-ERK 1/2 immunoreactivity showed an increased level after the treatment with PQ + MB HD and LD (127,8 / 118,35 ± 2 / 4,5 % and 107,33 / 128 ± 11,96 / 15,7 % of control, respectively) being statistically significant for ERK-1 after the association of PQ + MB HD. No changes was observed in P-ERK 1/2 in the CP after PQ + MB HD and LD (105,17 / 88,1 ± 21,58 / 23,96 and 93,33 / 67,67 ± 25, 44 / 18,35 % of control, respectively)(Fig 17). No significant changes was observed after the treatment with PQ

+ MB HD and LD in P-CREB (106,81 ± 13,99 % and 92,86 ± 5,38 % of control, respectively)(Fig 18) and GAD 65/67 (88,36 ± 8,41 % and 93,61 ± 3,4 % of control, respectively)(Fig 19) immunoreactivity in the SN. The treatment with PQ + MB HD and LD doesn't generates changes in values of P-CREB and GAD 65/67 (95,96 ± 5,53 and 101,28 ± 7,09 % of control, respectively) immunoreactivity in CP (Fig 18,19).

The values obtained in PDYN mRNA levels showed a decreased after the treatment with PQ + MB HD and LD both in the SN (69 ± 11 % and 91 ± 8 % of control, respectively), being statistically significantly after the treatment with PQ + MB HD. While in the CP no differences for both associations (93 ± 21 % and 99 ± 6 % of control, respectively) was observed (Fig 20). KOPr mRNA levels showed a significantly decrease after the treatment with PQ + MB HD, but not after PQ + MB LD (59 ± 4 % and 71 ± 6 % of control, respectively) in SN. A decrease was observed in the KOPr mRNA levels after PQ + MB HD and LD (66 ± 10 % and 91 ± 9 % of control, respectively) in the CP being significant only after the treatment with PQ + MB HD (Fig 21).

Regarding the N-OFQ/NOP system we observe increased levels of ppN/OFQ mRNA after the treatment with PQ + MB HD and LD (115 ± 21 % and 152 ± 16 % of control, respectively), being statistically significant only for the LD in SN. No variation in the values of ppN/OFQ mRNA expression in the CP for both associations (93,9 ± 19,4 % and 89,7 ± 10,5 % of control, respectively) was observed (Fig 22). Level of NOPr mRNA expression showed a statistically significant decrease in the SN after the treatment with PQ + MB HD and LD (63 ± 5 % and 63 ± 7 % of control, respectively), while in the CP, NOPr gene expression did not change significantly after PQ + MB HD (100,1 ± 12,2 % of control) and LD (94,2 ± 11,4 % of control) treatment (Fig 23).

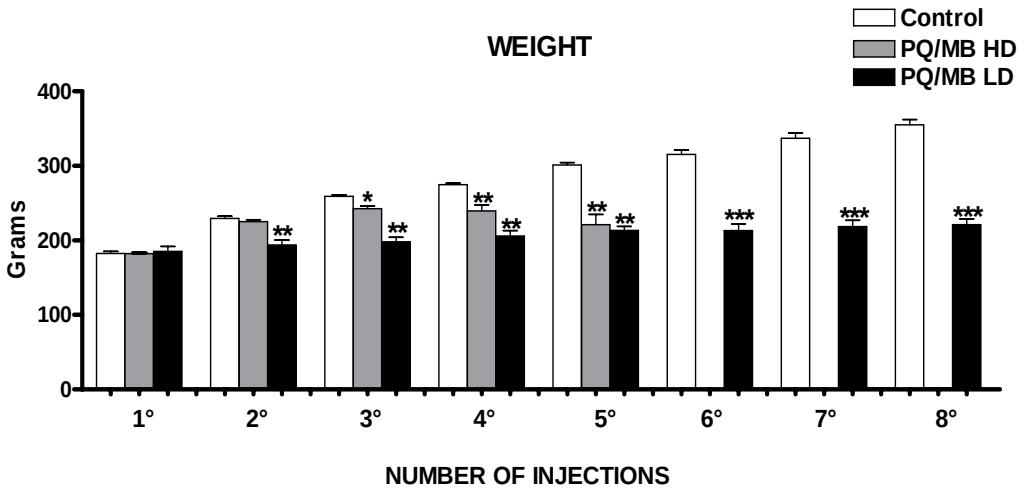


Fig 11.- Weight of rats after administration of PQ + MB HD and PQ + MB LD. *P < 0.05; **P < 0.01 versus control group in the same time of injection.

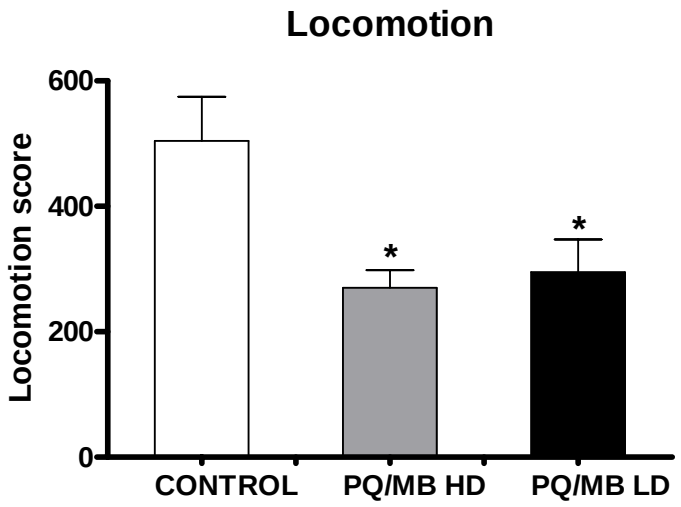


Fig 12.- Locomotion activity on the day after the last administration of PQ + MB HD and PQ + MB LD in rats. *P < 0.05 versus control group.

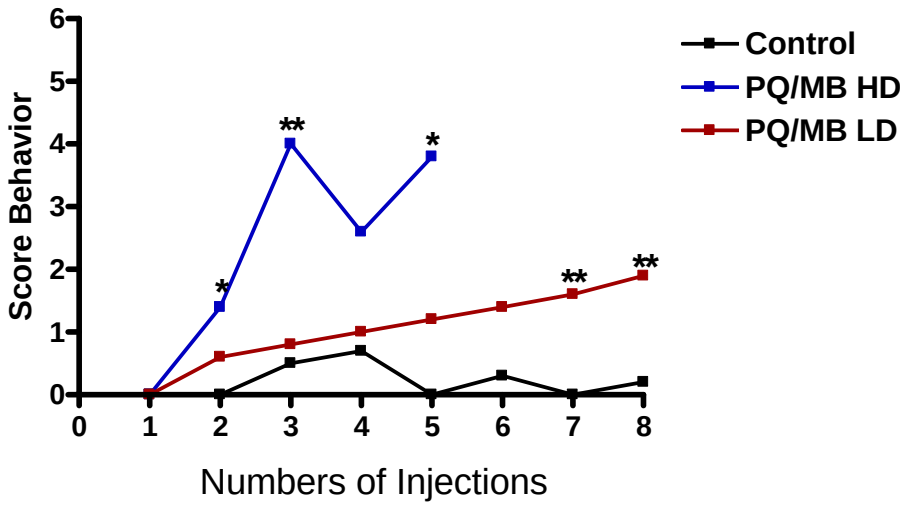


Fig 13.- Behavioral effects on the day after PQ + MB HD and PQ + MB LD administration in rats. *P < 0.05; **P < 0.01 versus control group.

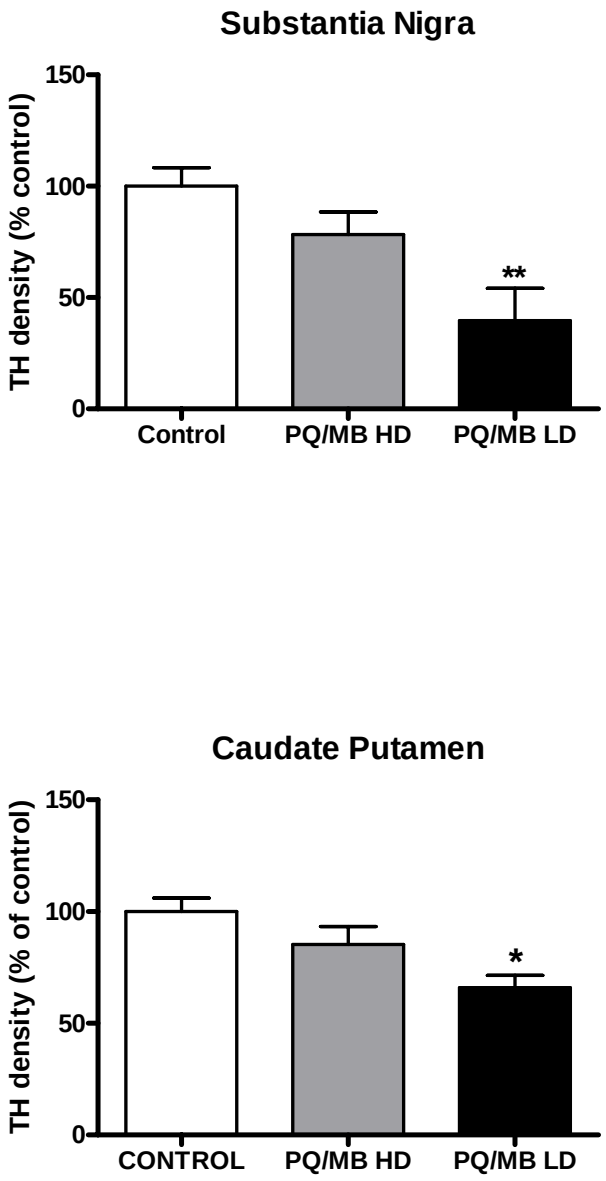


Fig 14.- Effects of PQ + MB HD and PQ + MB LD on the density of TH immunoreactivity in SN and CP. *P < 0.05 **P < 0.01 versus control group. Values represent mean ± SEM.

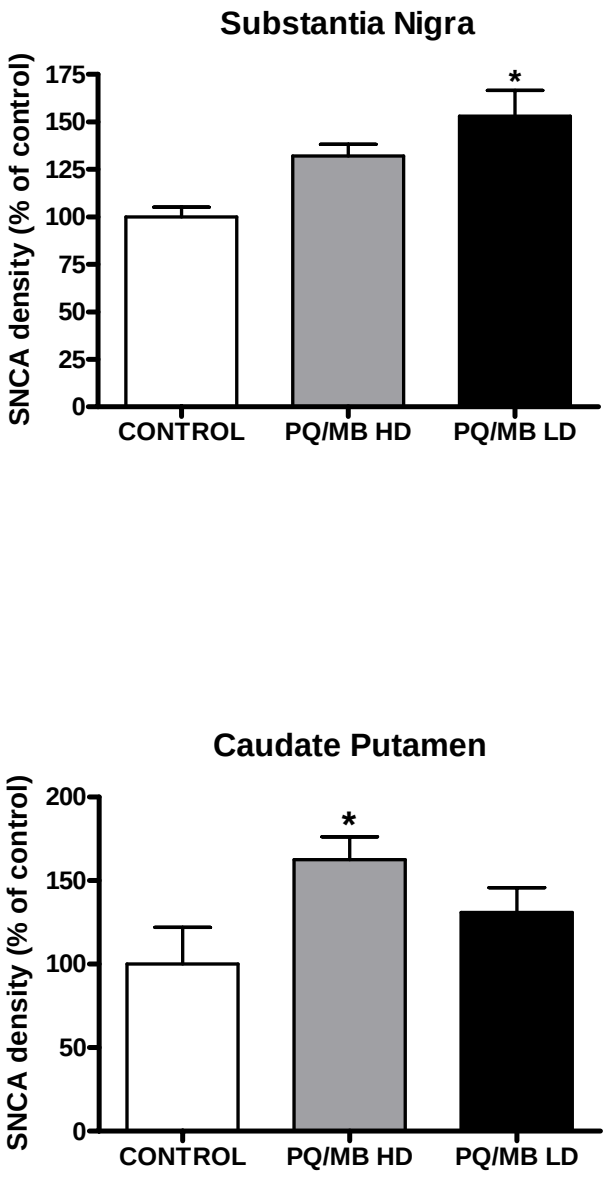


Fig 15.- Effects of PQ + MB HD and PQ + MB LD on the density of TH immunoreactivity in SN and CP. *P < 0.05 versus control group. Values represent mean ± SEM.

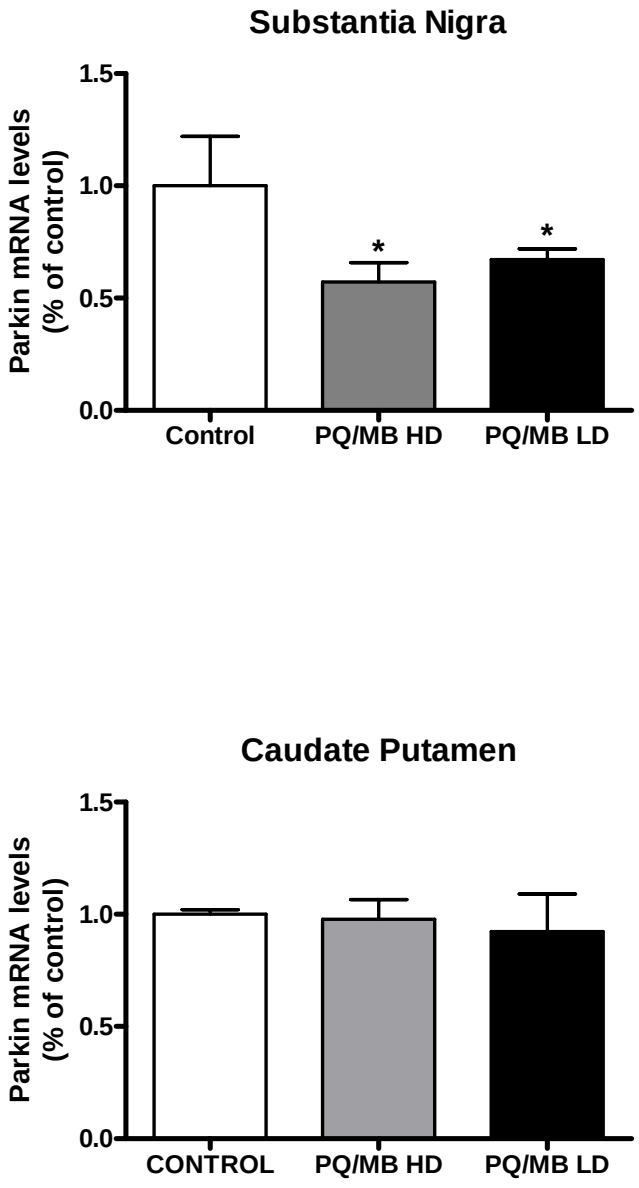


Fig 16.- Levels of Parkin mRNA in SN and CP. Bars represent 2^{-DDCt} value calculated by DDCt method. Expression was normalized to GAPDH and means of mRNA levels are expressed relative to control ± SEM. *P < 0.05 versus control group.

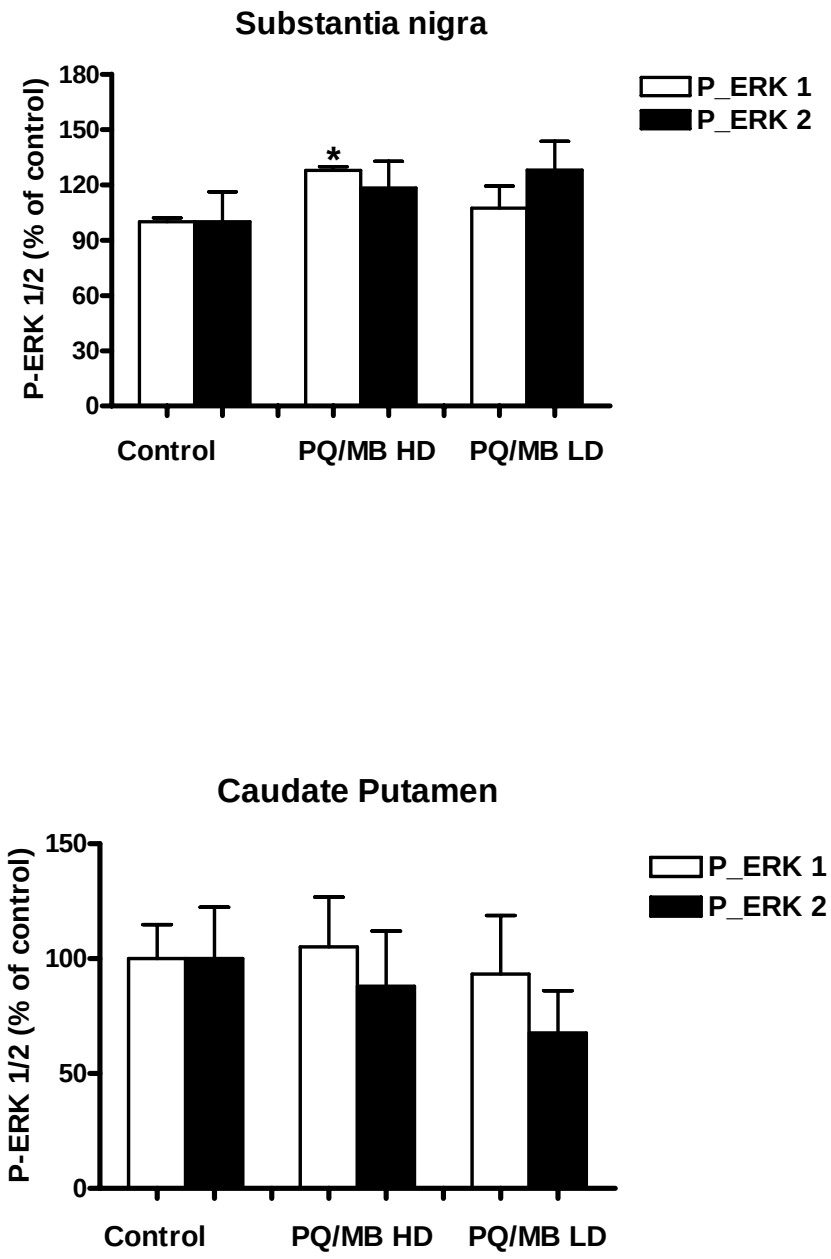


Fig 17.- Effects of PQ + MB HD and PQ + MB LD on the density of P-ERK 1/2 immunoreactivity in SN and CP. *P < 0.05 versus control group. Values represent mean ± SEM.

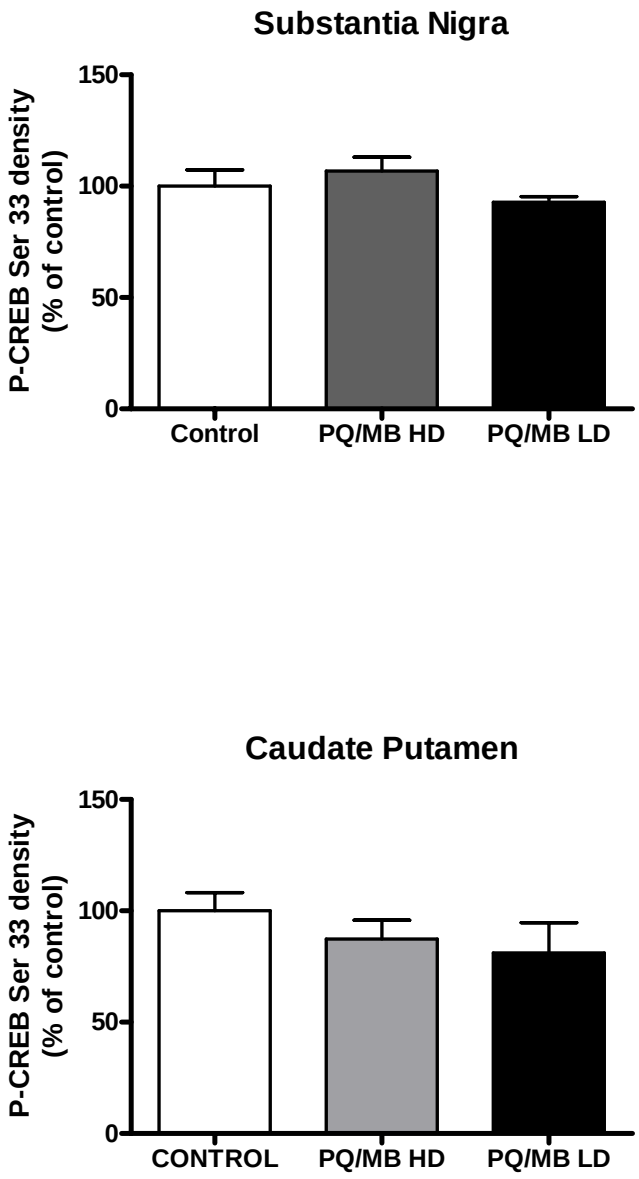


Fig 18.- Effects of PQ + MB HD and PQ + MB LD on the density of P-CREB immunoreactivity in SN and CP. Values represent mean ± SEM.

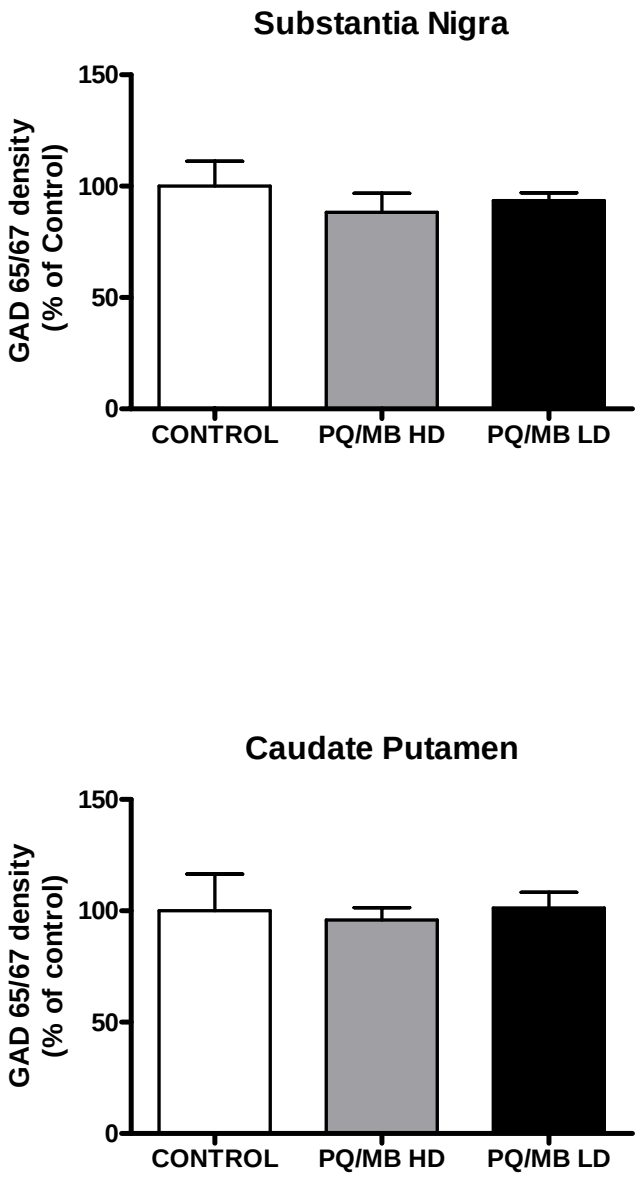


Fig 19.- Effects of PQ + MB HD and PQ + MB LD on the density of GAD 65/67 immunoreactivity in SN and CP. Values represent mean ± SEM.

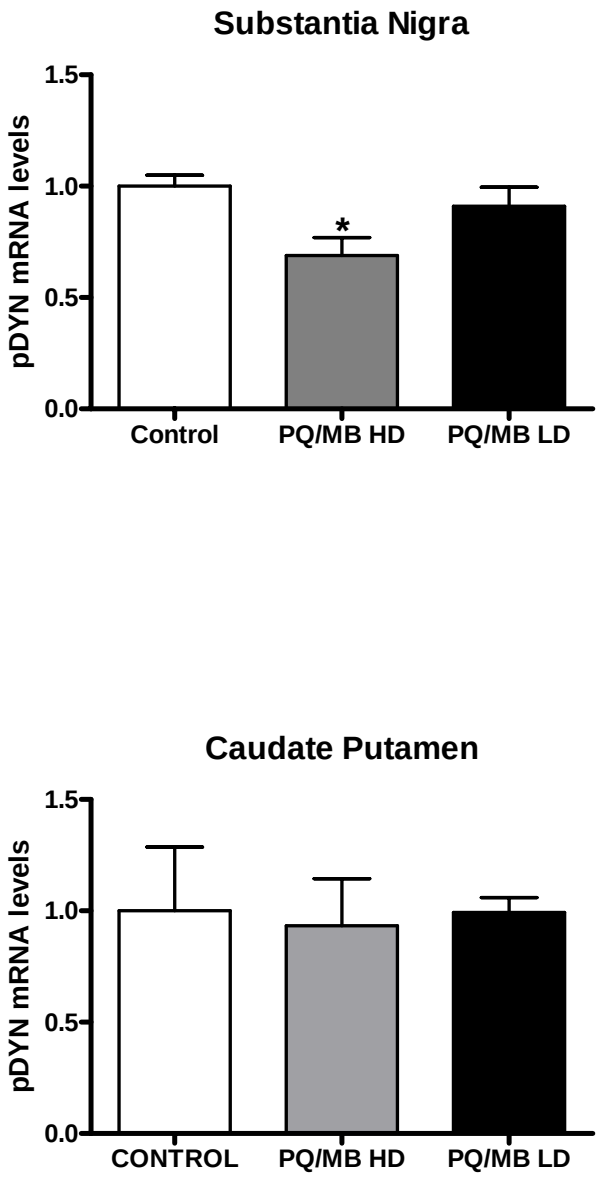


Fig 20.- Levels of pDYN mRNA in SN and CP. Bars represent 2^{-DDCt} value calculated by DDCt method. Expression was normalized to GAPDH and means of mRNA levels are expressed relative to control ± SEM. *P < 0.05 versus control group.

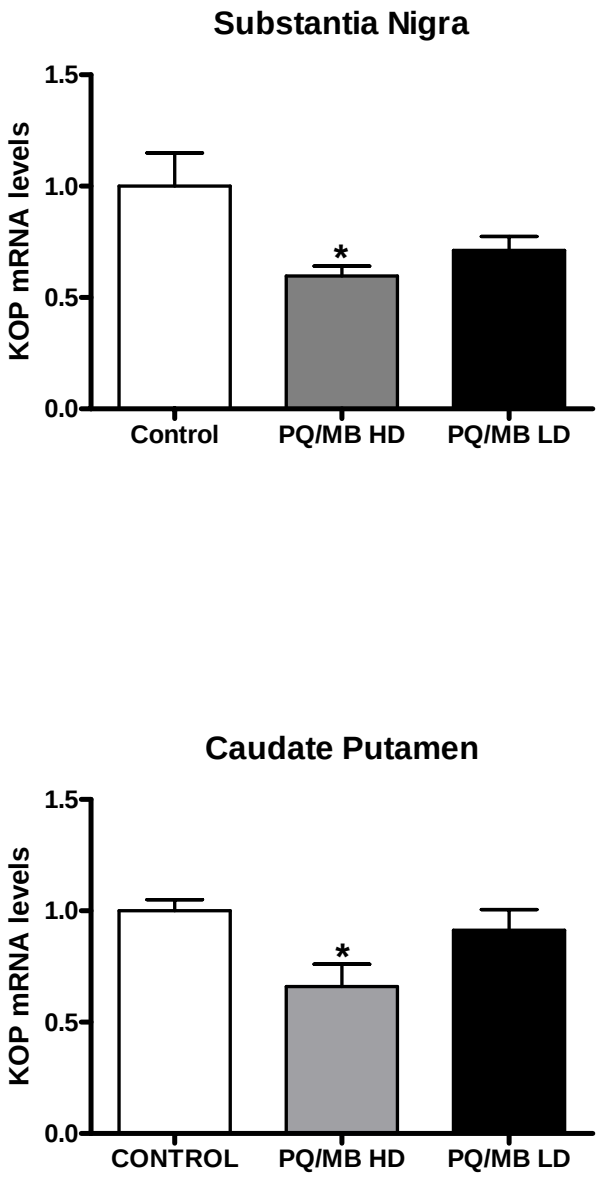
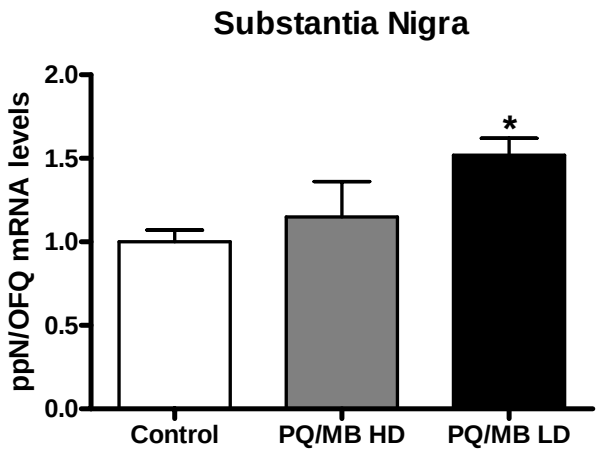


Fig 21.- Levels of KOP mRNA in SN and CP. Bars represent 2^{-DDCt} value calculated by DDCt method. Expression was normalized to GAPDH and means of mRNA levels are expressed relative to control ± SEM. *P < 0.05 versus control group.



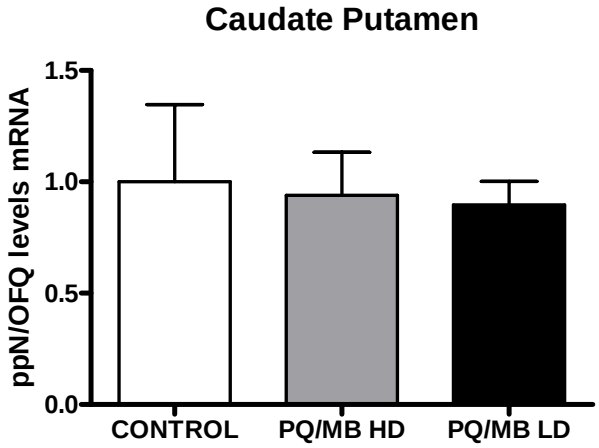


Fig 22.- Levels of ppN/OFQ mRNA in SN and CP. Bars represent 2^{-DDCt} value calculated by DDCt method. Expression was normalized to GAPDH and means of mRNA levels are expressed relative to control ± SEM. *P < 0.05 versus control group.

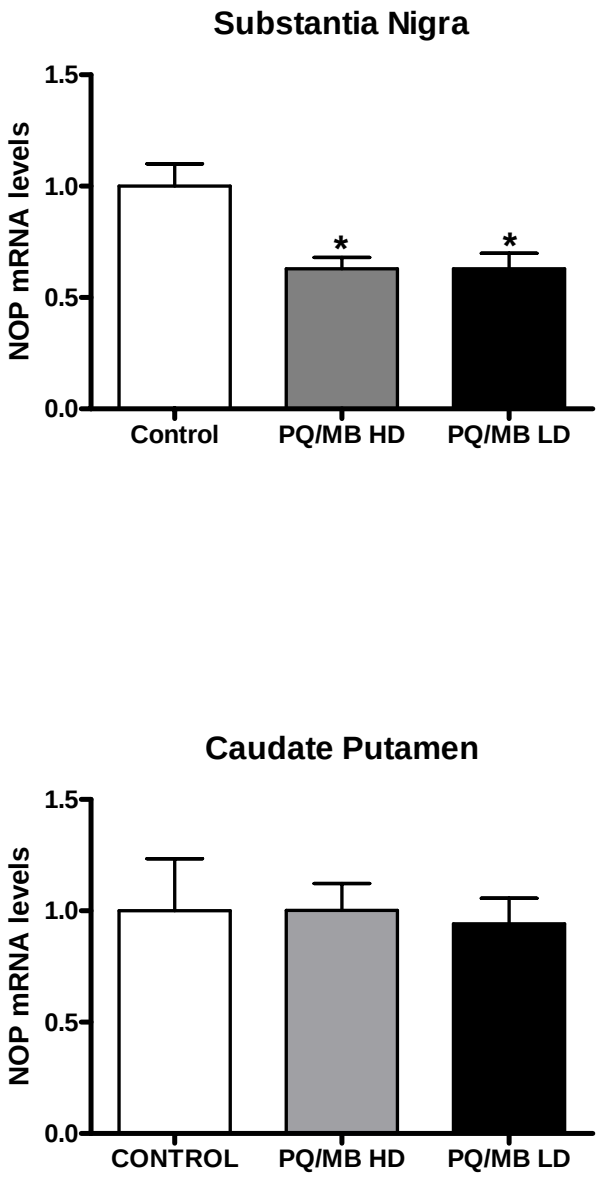


Fig 23.- Levels of NOPr mRNA in SN and CP. Bars represent 2^{-DDCt} value calculated by DDCt method. Expression was normalized to GAPDH and means of mRNA levels are expressed relative to control ± SEM. *P < 0.05 versus control group.

4.2 ALZHEIMER'S DISEASE

The gene expression analysis showed increased values for APP, SIRT1 and PIN1 in the AD twin, respect the healthy twin. No changes where observed beetwen the MZ twin for the APOE mRNA levels. A decrease in the values of PSEN1 mRNA was observed in the AD twin respect of control twin (Table 7).

Regarding the changes in DNA methylation, we observe a general decreased methylation level of APP, SIRT1, PSEN1 and PIN1 for the MZ twins (healthy and AD). No difference in methylation levels was observed in the promoter region of APOE (Table 8).

An increase in the histone modification that leading to activation of transcription was observed in the immunoreactivity of H3K9 Ac and H3K4 me3 (34,8 and 84,1 %, of control, respectively) in the AD MZ. While an increase in the density of H3K27 me3 (129% of control) was observed in the AD twin (Table 9). Representative immunoblots are shown in Fig 24.

	APP		SIRT1		APOE		PSEN1		PIN1	
MZ twins	Cont	AD	Cont	AD	Cont	AD	Cont	AD	Cont	AD
Gene expression	1	↑↑↑	1	↑↑↑↑	1	0,97	1	0,46	1	41

Table 7.-Gene expression analysis: The DDCt for each gene is indicated. The DDCt was calculated from the changes in gene transcription in the AD versus the healthy twin.

	APP		SIRT1		APOE		PSEN1		PIN1	
MZ twins	Cont	AD	Cont	AD	Cont	AD	Cont	AD	Cont	AD
% Met. DNA	2,3	5,8	1,1	3,8	100	100	0	0	2,9	0,9

Table 8.-DNA methylation status of gene promoter regions: % of methylation at each gene promoter calculated for AD and control (CT) twins using Myod as reference gene.

	H3K9 Ac		H3K4 me3		H3K27 me3	
MZ twins	Cont	AD	Cont	AD	Cont	AD
Immunoreactivity (%)	100	34,8	100	84,1	100	129

Table 9.-Analysis of histone modifications: H3K9Ac, H3K4me3, H3K27me3, % changes between AD and control (CT) twins.

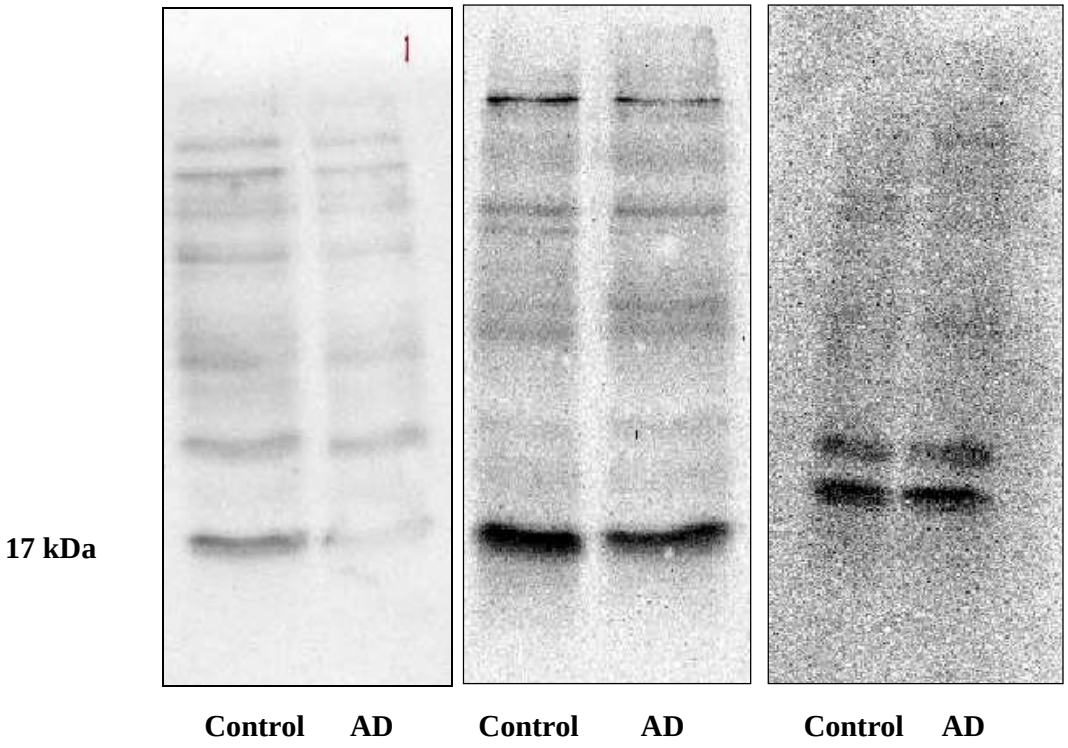


Fig 24.- Representative immunoblots for histone modifications, H3K9 Ac, H3K4 me3 and H3K27 me3 respectively.

5. DISCUSSION

5.1 PARKINSON'S DISEASE

In the present study, the 40% of the treated animals with PQ + MB HD either died or had to be sacrificed as a result of severe weight of loss and/or signs of respiratory distress following the third injection, consistent with progressive neurotoxicity (Cicchetti et al 2005, Saint-Pierre et al 2006). As pointed by Cichetti et al 2005, who established that differently to other pesticides, the association of PQ + MB do not result in animal death, our study indicates that the association of PQ + MB LD generated a consistent changes in weight with weight increase but at a lower rate than control group. Cichetti et al 2005 have also indicated that animals treated with PQ or the association of PQ + MB show respiratory difficulties due to lung anormalities like alveolitis and broncheolitis. This observation agrees with our results, since we have evidenced respiratory distress in animals treated with the associations of PQ + MB HD. It has been described that only combined exposure was able to produce the sustained decreases in motor activity immediately following injections, with activity levels returning to control values 24 h later (Thiruchelvam et al 2000). These results associated with behavior demonstrated that both pesticides are capable to generate changes in the neural connexion between SN and CP, essential brain regions that support the normal motor functions (Dinis-Oliveira et al 2006).

A decreased immunoreactivity of TH, the rate-limiting enzyme for dopamine synthesis, was observed in both brain areas indicating progressive loss of dopaminergic neurons, with a lower TH levels after the treatment with PQ + MB LD in SN, confirming dopaminergic system damage. We can hypothesize that the type of exposure, like dose and number of injections can modulate different levels of degeneration, indicating that a lower dose but at major time of exposure can induced a hight loss of dopaminergic cell, confirmed by a significant decreased of TH immunoreactivity in both brain areas after the lower dose.

As previously mentioned one of the histopathological hallmark of PD is the presence of Lewy bodies. One theory is that Lewy bodies could be beneficial to neurons, moving the partially aggregated species into a relative safe and sequestered form (Baptista et al

2004). The increase of SNCA observed in our study is in agreement with other studies, *in vivo* and *in vitro* (Manning-Bog et al 2002) revealing that the fibrillation of SNCA may be accelerated by common pesticides such as PQ and MB (Manning-Bog et al 2002) causing oxidative damage and leading to a higher cells vulnerability (Hsu et al 2000, Kanda et al 2000, Ko et al 2000). Also, other authors have shown that dopamine can modulate the aggregation of SNCA. Dopamine induces the aggregation of both A53T and WT SNCA in human neuroblastoma M17 cells (Moussa et al 2008). SNCA expression can alter the homeostasis of dopamine leading to an increase in cytosolic levels; the oxidation of dopamine generates reactive intermediates and ROS that among other things causes impairments of protein function (Leong et al 2009). For otherwise was observed a decrease in mRNA levels of parkin. Parkin is neuroprotective in a number of different model systems and, importantly, can protect against SNCA toxicity in vitro (Petrucelli et al 2002) and in vivo (Yang et al 2003). This leads to the concept that parkin and SNCA have opposite actions and affect cell survival pathways that may involve the toxicity of cellular proteins (Baptista et al 2004). Despite the observation that SNCA has a major effects on the mitogen-activated protein kinase (MAPK) and stress-activated protein kinase pathways, and for this reason the overexpression of SNCA can result in an inappropriate activation of the MAPK pathway throught interaction between SNCA and ERK-2, our results indicate only changes in the values of P-ERK-1 on the SN in the animals treated with PQ + MB HD, but not in the rest of the treated animals, suggesting that in our experimental conditions the pesticides utilized did not involve these pathways in the mechanisms underlying the development of PD symptoms. No change was observed in the values of P-CREB in the SN and CP of all treated animals, suggesting that the toxicity of PQ and MB has not involved this way of action. To determinate the effect of PQ + MB, in the non-dopaminergic neurons (i.e. GABA), we assessed the levels of GAD 65/67, enzyme that convert glutamate to GABA. In our experiment the levels of GAD 65/67 were unmodified after both treatments in SN and CP, supporting a lack of involvement of PQ + MB toxicity in the GABAergic system. These results are in agreement with our previous data (Di Benedetto et al 2009), obtained after MPP+ administration.

We observed a significant decrease of pro-dynorphin (PDYN) mRNA levels in SN of animals treated with PQ + MB HD, and a significant decrease in the levels of KOPr was obtained in the SN of rats after the treatment with PQ + MB HD. A large body of work,

based on animal models of PD, has demonstrated that dopamine denervation modulates the expression of striatal peptides (Steiner and Gerfen 1998, Carta et al 2001, Carta et al 2003). Removal of DA input to the striatum leads to a decrease in dynorphin mRNA expression and increased levels of enkephalin mRNA (Steiner and Gerfen 1998, Carta et al 2001, Carta et al 2003). The imbalance between these two pathways is thought to contribute to the motor dysfunction associated with PD. Animals treated with PQ + MB HD showed a compromised motor activity thus the modifications observed in KOPr mRNA after this treatment could affect motor compromission. PDYN and KOPr are localized in abundance in both globus pallidus and SN reticulate (Haber and Watson 1985, Mansour et al 1987) placing this opioid system in a strategic location to modulate motor function. Even thought data on PDYN in PD are not unequivocal, a decrease in the PDYN levels in the striatum has been observed in PD patients (Bäckman et al 2007). Nevertheless PD is a chronic neurodegenerative disease normally associated with aging; it cannot be easily compared with animal models of parkinsonism, which normally involve young animals with a rapid onset and short duration of DA neuron degeneration and striatal DA depletion. Others factors may influence the data on this system, like duration of treatment, affecting the neuromodulation activity and expression of striatal peptides (Bäckman et al 2007).

The possible role of N/OFQ-NOP system in the pathology of PD remains poorly understood, but it has been suggested that endogenous N/OFQ may contribute to symptoms and neurodegeneration associated with PD (Marti et al 2005, Di Benedetto et al 2009). The analysis of ppN/OFQ showed an increase in the mRNA levels in SN after the treatment with PQ + MB LD in according with other studies (Marti et al 2005, Di Benedetto et al 2009) with no significant differences observed in CP after either treatment. Several studies demonstrated that the i.c.v. administration of N/OFQ in rats decreases the extracellular dopamine levels in the SN and in the ventral tegmental area (Murphy and Maidment 1999), with some neuropathological implications that involve the control of motor activity due to the inhibitory action of N/OFQ on the release of several neurotransmitters, that include noradrenaline, serotonin, glutamate and dopamine (Schlicker and Morari 2000, Flau et al 2002). The default of alterations of GAD65/67 levels in either the SN or the CP following PQ + MB HD and LD administration, leads us to hypothesize a lack of involvement of glutamate in the toxicity of both pesticides. Moreover it has been shown that MPP+ induces an increase

in ppN/OFQ gene expression in the SN in the absence of significant changes in GAD65/67 expression, suggesting that even in the toxic effects of this neurotoxin there is an involvement of the N/OFQ–NOP system that may be evoked by a mechanism of action that does not require changes in GAD expression (Di Benedetto et al 2009). On the other hand, the effects of both treatments induce a significant 40% decrease in the values of NOP mRNA levels in the SN and CP. Our results are in agreement with data obtained in other experimental PD models, after 6-OHDA (Norton et al 2002) and with MPP+ treatments (Di Benedetto et al 2009), the latter compound having a structure similar to PQ. On the basis of these results, it has been suggested that the pharmacological or genetic blockade of NOP in the SN may represent a novel target in PD therapy (Marti et al 2005). Norton et al 2002 indicated that NOP mRNA is mainly expressed in tegmental and nigral dopaminergic neurons and, for this reason, the decrease in the values of NOP mRNA in the SN may reflect a loss of dopaminergic neurons, being consistent with the decrease of immunoreactivity of TH. On the contrary, N/OFQ mRNA is largely expressed in GABAergic neurons (20-25%) present in the SN, and this different localization may explain why the levels of N/OFQ were not decreased in this area. On the other hand, it is interesting to point out that we also observed a down-regulation of both ppN/OFQ and NOP mRNAs in the CP after both the neurotoxins administration. The cellular location and functional role(s) of ppN/OFQ and NOP mRNAs in the CP are unknown. Another possible explanation for these opposite results, between peptide and receptor, is that the NOP down-regulation observed might be a result of the increase of N/OFQ in the SN (Di Benedetto et al 2009).

Several publications have established that inhibiting N/OFQ-NOP system may improve the symptomatology of PD (Marti et al 2005, Marti et al 2007, Brown et al 2006).

5.2 ALZHEIMER DISEASE

MZ twins constitute an excellent example of how genetically identical individuals can exhibit differences and therefore provide a unique model to study the contribution of epigenetic modifications in the establishment of the phenotype.

Among the genes investigated, we observed a large increase in APP, SIRT1 and PIN1 gene expression in the AD versus the healthy twin, in agreement with previous data on APP overexpression in specific areas of AD brains (Johnson et al 1990). The increase observed in APP mRNA levels is in agreement with the pathologic changes presented in this disease, like formations of senile plaques, hallmarks of AD. Regarding the levels of PIN1, it has been demonstrated that it acts on both *tau* and APP, regulating their dephosphorylation, processing and biological functions (Liou et al 2003). The hyperphosphorylation of *tau* protein could be related to unbalanced kinase or phophatase activities, which are further regulated by other proteins like PIN1, leading to a tangle formation and neurodegeneration (Lu et al 2003). It has been hyphotesized that reduced PIN1 activity in the frontal cortex of patients with MCI contribute to the initial accumulation of hyperphosphorilated *tau* and followed, in a more advanced stage of the disease, by a compensatory upregulation of the PIN1 gene that counteracts Aβ plaque formation (Wang et al 2007). According to our results, Arosio et al 2011 have recently indicated that in PBMCs of subjects with late onset disease, a significant increase in PIN1 gene expression together with a significant decrease in gene promoter methylation was observed.

The induction of SIRT1 mRNA levels may be alterated under various neurotoxic conditions (Kim et al 2007) and may be interpreted as a neuroprotective adaptation response. It seems that SIRT1 enhance cellular repair mechanisms and buys time for these mechanisms to work, so conceptually sirtuins may promote health and longevity, in part, by slowing cell death and prolonging function in cells. Wang et al 2010 suggest that SIRT1 activation may downregulate the generation of Aβ peptides interfering with AD mechanisms. A possible link between SIRT1 and AD came from the potential benefits of caloric restriction on AD symptoms and progression. The epidemiology of neurodegenerative diseases are related with multiple genetic factors, diet and social

behaviour (Mattson et al 2002). High caloric diets are associated with the risk of AD, and a low caloric diet have a reduced risk of developing AD by promoting SIRT1-mediated regulation of APP processing mechanisms relevant to the generation of amyloidogenic Aβ peptides and neuritic plaque deposition in the brain in mouse model of AD (Wang et al 2010).

A strong association between early onset AD and high levels of PSEN1 expression has been described due the proteolytic activity of PSEN1 against βAPP, cleaving it and leading to Aβ and p3 accumulation (Steiner et al 1999). However, Theuns and Van Broeckhoven 2000 suggest that PSEN1 may be up or down regulated in AD depending on the cell-type analyzed. Our results confirmed this situation, since we found a decrease in the gene expression of PSEN1, suggesting that, even when the role described for PSEN1 is fundamental in Aβ generation, it is not the only one that could be proposed for this gene, mainly because of the existence of several cross-talk with another cellular pathways, like NOTCH signaling as suggested by Steiner et al 1999, which alternatively can induce cellular death leading to the disease presentation.

The presence of the ε4 allele in APOE represents the most important genetic risk factor for late onset sporadic AD, but despite the magnitude of the APOE ε4 risk effect and a possible mechanistic link with Aβ pathology, it is still far from clear how APOE ε4 is involved in AD pathogenesis (Kok et al 2009). The APOE protein plays a central role in the regulation of cholesterol and triglyceride metabolism in the context of AD pathology (Bales 2010). Moreover, it has previously proposed that the synthesis of ApoE might play a role in regional vulnerability of neurons in AD. Despites of these hypotheses, no changes was observed in APOE mRNA levels between the MZ twins, suggesting that several factors can influence the expression of this gene, and further studies are necessary to confirme the transcription of APOE gene.

With the getting age of the population, the growing incidence and prevalence of AD increases. It is clear that aging and AD are associated with epigenetic dysregulation at various levels, and twin studies in AD support the notion that epigenetic mechanisms mediated the risk for AD. Recently Wang et al 2008 reviewed evidence for non-mendelian anormalies in sporadic AD, suggesting an important role for environmental risk factors and epigenetic regulation in the causes of sporadic AD. The methylation of

CG sequence can affect nearby gene expression and hypomethylation of regulatory sequences usually relates to gene expression, in agreement with the majority of our results for DNA methylation of promoters regions, where a very low hypomethylation was observed for many of the genes investigated in both MZ twins, even for those where alterations on gene expression were observed.

The hypomethylation observed in APP promoter region seems to have again a strong component. One study from the early 90's in a post-mortem brain sample of an unaffected patient suggest that APP promoter is always unmethylated in brain and hence may not be controlled by DNA methylation in the brain of healthy individuals (Milici et al 1990). These results may be confirmed by a low DNA methylation in the APP promoter region close to the transcriptional start site reported in both AD and healthy subjects in post-mortem analysis of the frontal cortex and hippocampus of the promoter for APP and PSEN1 (Barrachina et al 2009). Age-dependent methylation changes associated with AD have been previously reported for the APP gene. Toghi et al 1999 reported that some of the CpG sites within the APP promoter can be partially methylated in brains of healthy individuals (≈26% methylation), accompanied by a reduction with age (<8%) in methylcytosine content in these CpG sites.

Moreover, a hypomethylation of PSEN1 that was not related to age or disease state has been documented (Siegmund et al 2007). PSEN1 is expressed at high levels in the brain cells as well as in lymphocytes and are, as expected, unmethylated in both tissues (Wang et al 2008). The same authors described in prefrontal cortex of late onset AD an abnormal PSEN1 methylation patterns and were usually associated with hypomethylation of the promoter.

The hypomethylation observed for the PIN1 promoter region is in agreement with the findings in PBMCs of subjects with late onset AD, where observed a significant increase in PIN1 gene expression together with a significant decrease in gene promoter methylation (Arosio et al, 2011). The authors suggest that the modifications found in PIN1 in the same subjects support the hypothesis that PIN1 plays a signifanct role in AD.

APOE is the main candidate for late onset AD and patients are routinely screened for APOE genotypes. We found that APOE gene was strongly methylated in both twins, complementing a previous study in both late-onset AD and normal elderly (Wang et al 2008). It is important to point out that both twins were carring the ApoE ε4 allele, a major genetic risk factor for late-onset sporadic AD (Bu 2009). No difference on promoter for APOE was observed, but is possible to observe a major level of methylation than the other genes. Interestingly, the APOE gene belongs to a group of genes that no posses a classical CpG island in their promoters, but rather low CpG density regions (Weber et al 2007).

Overall, our results are in agreement with a generalized reduction of DNA methylation upon ageing (Scarpa et al 2006). Several researchers have reported both global and locus-specific differences in DNA methylation; and histone acetylation in identical twins at different ages (Fraga et al 2005). They have concluded that, whereas young MZ twin pairs are essentially indistinguishable in their epigenetic markings, older MZ twin shows substantial variations, consistent with changes in gene expression, and the differences reported is up to four times greater in older MZ twins than those observed in young twin couples (Martin 2005).

Data on histone modifications in humans AD brain tissues are sparse. It was reported, in a post-mortem neuropathological examination of the brains of MZ twins discordant for AD, that the pathology of the disease was associated with a marked increase of the H3K9 me3, considered as marker of gene silencing, and condensation of heterochromatin structure in the temporal cortex and hippocampus of the twin with AD when compared with the healthy twin (Chouliaras et al 2010). Interestingly, among the histone modifications studied it has been observed a strong reduction of H3K9Ac, tipically associated with gene activation, in the AD twin**.** The latter finding seems of particular relevance in the context of published data, since the observed up-regulation of SIRT1 could be linked to an increase of histone deacetylases and a subsequent reduction of histone acetyltransferases (Narayan and Dragunow 2010). In accordance with our results, it has been reported an increase in the levels of SIRT1 evidenced by a decrease in acetylation state of PGC-1 alpha, a target for SIRT1 deacetylase activity (Nemoto et al 2005). The decrease in the immunoreactivity observed for H3K9 Ac and H3K4 me3

and the increase in the density of H3K27 me3 allows hypothesize a general low gene expression for AD.

6. CONCLUSIONS AND PERSPECTIVES

Our results allow us to conclude that the exposure to PQ and MB by action of DA modulates the N/OFQ–NOP system gene expression in SN and CP, strengthening the hypothesis that this neuropeptidergic system could be strongly involved in the mechanisms underlying PD. Furthermore, our findings enhance the evidence available, supporting the hypothesis of a potential value of NOPr antagonists for the alleviation of PD symptoms, and possible neuroprotection following exposure to neurotoxins.

On the other hand, the results regarding AD help to improve our understanding of key-genes in AD. APP, APOE, PSEN1, SIRT1 and PIN1 are involved in AD pathogenesis, as the main theories has pointed, and epigenetic changes related to promoter region of these genes changes according pathologic status. Additionally, these results allow confirm that the use of peripheral blood cells is a useful model for the study of gene regulation directed to identify critical alterations within the brain. Finally, this study also suggests that epigenetic differences, possibly related to environmental factors and normal physiology, could be relevant to generate molecular alterations linked to AD.

Diagnosis of Parkinson's and Alzheimer's diseases is mainly made by exclusion of other neuropathologies with similar symptomatologie. Moreover, the impossibility to access directly to the affected tissue prevents identify the first critical changes that verify in the brain at the beginning of the disease. Traditionally, animal models have allowed improve our knowledge about these diseases, evidencing main pathways involved and suggesting potential therapies. However, they are only models and lack of some important keys related with these diseases. Epigenetic modifications have proven to be related with changes of gene expression in several pathologies and these modifications can be detected in PBMCs.

In conclusion, the results of this doctoral thesis support the idea that epigenetic changes assessed in PBMCs can be useful in neurodegenerative disorders, like AD and PD, enabling identification of new biomarkers in order to develop early diagnostic programs.

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ADDENDUM

Lavori in extenso

1. Di Benedetto M., Bastías Candia S., D'Addario C., Porticella E.E., Cavina C., Candeletti S., Romualdi P. Regulation of opioid gene expression in the rat brainstem by 3,4-methylenedioxymethamphetamine (MDMA): role of serotonin and involvement of CREB and ERK cascade. Naunyn-Schmied Arch Pharmacol (2011) 383:169–178.

2. B.Arosio, A.Bulbarelli, S.Bastias Candia, E.Lonati, L-Mastronardi, P.Romualdi, S.Candeletti, C.Gussago, D.Galimberti, E. Scarpini, B.Dell'Osso, C.Altamura, M. Maccarrone, L.Bergamaschini, C.D'Addario and D. Mari. Pin1 contribution to Alzheimer's (AD) disease: transcriptional and epigenetic mechanisms in patients with late-onset AD (accepted for publication *Journal of neurodegenerative disease*) doi: 1 O. 1 1591000333799.

Comunicazioni a congressi

1. Bastias Candia S. Nociceptin/Orphanin system, α-synuclein and parkin gene expression in animal models of Parkinson's disease, XIV Seminario Nazionale per Dottorandi in Farmacologia e Scienze affini, Siena, Certosa di Pontignano, 20-23 Settembre 2010.

2. S. Bastias Candia, C. Abbate, A. Casè, C. D'Addario, S. Candeletti, P. Romualdi, B. Arosio, C. Vergani, L. Bergamaschini; "Valutazione dell'espressione di geni selezionati in una coppia di gemelle omozigoti discordanti per la malattia di Alzheimer" Presentation in 55° Congresso Nazionale SIGG dal titolo "INVECCHIAMENTO E LONGEVITA': PIU' GENI O PIU' AMBIENTE? Florence (Italy) 2010.

3. Di Benedetto M, D'Addario C, Dell'Osso B, Bastias Candia S, Cortini F, Galimberti D, Scarpini E, Candeletti S, Altamura A, Romualdi P; "Invenstigation on DNA methylation status of opioid peptides promoters in PBMCs of subjects

with bipolar disorders", Poster in 8th meeting of the European Opioid Conference, Krakow (Poland) 13th-15th April 2011.

4. Sussy del C. Bastías Candia, Manuela di Benedetto, Claudio D'Addario, Sanzio Candeletti and Patrizia Romualdi; "Role of Nociceptin/Orphanin system in Paraquat and Maneb animal model of Parkinson's disease" Poster in IBRO Congress, Florence (Italy) 2011.

5. Bastias Candia S. Role of Nociceptin/Orphanin system in paraquat and maneb animal model of Parkinson's disease, XV Seminario Nazionale per Dottorandi in Farmacologia e Scienze affini nell'ambito del 35° Congresso Nazionale della Società Italiana di Farmacologia, Bologna, 14-17 Settembre 2011.

6. Di Benedetto M., Bastias Candia S., D'Addario C., Candeletti S., Romualdi P. 3,4-methylenedioxymethamphetamine (MDMA) regulates opioid gene expression in the rat brainstem through the involvement of serotonin and the activation of CREB and ERK cascade. 35° Congresso Nazionale della Società Italiana di Farmacologia, Bologna, 14-17 Settembre 2011.