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Neurodegenerative diseases: molecular mechanisms and new strategies for neuroprotection

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Preface

The Neurodegenerative process

The term neurodegeneration defines a variety of conditions that modify neuron's normal functions in the human brain in which is possible to observe a progressive and consistent neuronal loss. Neurons are cells that form the central nervous system (CNS) and they are characterized by inability to reproduce or replace themselves. In this way, when damage threatens, the loss of cells will be permanent. The definition of neurodegenerative disease includes a multitude of diseases which could be separated into two main groups, chronic and acute pathologies (1).

The mechanisms involved in the evolution of these superclass are not completely understood yet, however they share common characteristics such as misfolded proteins, oxidative stress, inflammation, excitotoxicity and, obviously, neuronal loss (2).

Neurodegenerative chronic diseases are estimated to surpass cancer as the most important cause of death by the 2040s (3). This definition identifies a variety of pathologies such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, amyotrophic lateral sclerosis (ALS) and so forth, which share many common characteristics like oxidative stress, microglia dysfunction and progressive neuronal loss (4). An extensive variety of these pathological conditions are characterized by proteins accumulation like intracellular or extracellular aggregates (5). This process can occur both in sporadic and familial cases, that highlights this pathological mechanism strongly correlated with neuron dysfunctions (6). Usually, patients are affected later in life, for this reason, in the last 20 years the number of diagnosis is prominently enhanced, especially for the increase in life expectancy. However, numerous evidences suggest the presence of a

preclinical asymptomatic stage that could begin years before the establishment of the first symptoms. This stage could be the most important phase because represents the time point where is possible to modulate the establishment of the disease (7). Patients could present a variety of symptoms that frequently occurs when the pathology in CNS is already advanced. The current therapy could not restore the physiological central condition, but could only operate on the modulation of somatic symptoms and, in the best situations, try to slow down the progression of the neurodegenerative process without arresting it (8).

On the other hand, the term acute neurodegeneration describes clinical conditions in which neurons are rapidly damaged and usually die in response to an insult. This description comprehends stroke, head injury, cerebral or subarachnoid hemorrhage, and ischemic brain damage derived from fetal or perinatal hypoxia. These conditions cause massive morbidity and mortality, and pose an enormous socio-economic burden. Although the type of insult and onset of neuronal injury is acute, subsequent neuronal loss can occurs hours or days after the initial event. This delayed cell damage results from endogenous factors that are released in response to the primary injury, which might be common to the clinical conditions outlined.

Inflammatory processes have been implicated in both acute and chronic neurodegenerative conditions (9). The CNS differs in its inflammatory response to other tissues. In general terms, cellular infiltration in the brain in response to inflammation, infection and injury is weaker and delayed, but many inflammatory responses can be induced rapidly (10). These include the activation of microglia, and the expression and release of classical inflammatory mediators, such as acute-phase proteins, eicosanoids, complement and cytokines (11).

Many studies have shown the frequency to develop a neurodegenerative chronic disease several years after an acute brain injury (12). In addition, many patients show, after a traumatic brain injury, motor

and cognitive manifestations that are close to which are observed in neurodegenerative chronic patients. For this reason, in preclinical research, it is frequently used a battery of behavioral tests similar to those employed in PD or AD studies. These considerations are combined to the fact that neuronal death can be caused by protein misfolding, neuroinflammation and oxidative stress; these mechanisms are shared by these two different superclasses of neurodegenerative diseases. Moreover, it is possible that a neuroprotective strategy may be aimed to arrest or slow down the damage evolution in both chronic and acute neurodegeneration should act in the window of time between the lesion occurred and the propagation of the irreversible injury.

In this context it is evident the importance of understanding how it is possible to modulate all these mechanisms and to work on the connection between them from the moment that they share a common base. For this reason it is evident how is fundamental the concept of neuroprotection as a way to modulate and control the evolution of neurodegenerative processes. Neuroinflammation, oxidative stress and the apoptotic process may be a functional targets where operate to this end.

Taking into account these considerations, the aim of the present study is to identify potential common pathogenetic pathways in neurodegenerative diseases using an integrated approach of preclinical studies. The goal is to delineate therapeutic strategies for the prevention or reduction of neuroinflammation, neurodegeneration and cognitive dysfunction associated to PD and cerebral ischemia. The results may contribute to the development of pharmacological strategies designed to prevent and counteract neurodegenerative damage at morphological functional, biochemical and cognitive levels.

PART I

1. INTRODUCTION

1.1. PARKINSON'S DISEASE

In 1817 James Parkinson described, in his monograph "Essay in the Shaking Palsy", the essence of the second most common age-related neurodegenerative disorder after AD, afflicting approximately 6 million people worldwide (2). Parkinson's disease is a multisystemic slowly progressive disorder characterized by a combination of motor and non-motor symptoms. The main pathological change in the parkinsonian brain is the degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNpc) in the ventral midbrain and depletion of dopamine in the striatum (STR), principal site where these nerve terminals project. Furthemore the condition is associated with an accumulation of neuronal inclusions known as Lewy bodies (LBs) (13) (Fig. 1).

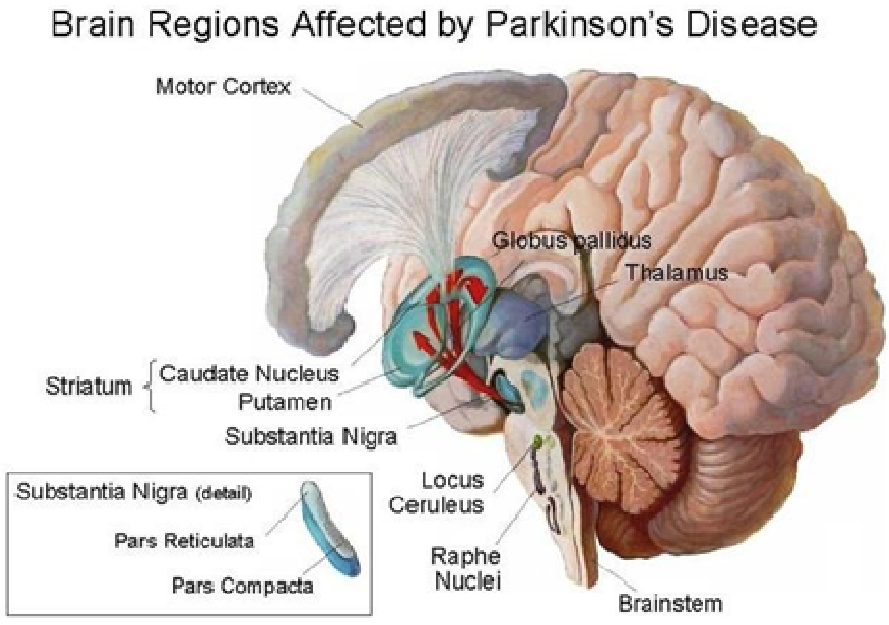


Figure 1. Brain Regions involved in Parkinson's disease.

PD is characterized by cardinal motor symptoms, including bradykinesia, rigidity, postural instability, and resting tremor. In addition to motor symptoms, PD patients also show, during the early stage, non-motor manifestations such as autonomic dysfunctions, cognitive abnormalities, sleep and mood disorders, pain, and sensory conditions (14). The complexity of the pathology makes many difficulties to find the cause of the disease and to recognize which is the moment when it starts. This is because of the long period between the initiation of the disease and the manifestation of clinical symptoms; not less important is the lack of any distinctive blood biomarker with which to trace the disease process. Moreover, clinical symptoms appear when almost 50% of pigmented dopaminergic neurons are dead. In this condition, surviving cells could supply the striatum demand only about 20-30% and first motor symptoms start to be evident (15). This combination of numerous motor and non-motor symptoms makes PD as a unique chronic condition with a lot of questions still unanswered. Motor disabilities could restrict patient's activity and the association with non-motor symptoms could importantly worsen their quality of life (16).

1.1.1.INCIDENCE AND EPIDEMIOLOGY

PD is a progressive disease with a mean age at onset of 55, the pathology's prevalence rises to 1% in persons 65 years of age, this percentage arrives to 3% in individuals with an age over 85 without significant difference by the sex (17). In about 95% of cases PD is idiopathic multifactorial disease due to environmental factors and genetic susceptibility, there is no apparent familiar genetic connection; these cases are referred as "sporadic" PD. On the other hand, leftover 5% cases are the result of genetic mutations and are inherited (2). Usually the pathology is diagnosed around 60 years of age, but in 10% of cases the onset would be really earlier around 45 years, this is considered by the scientific community like precocious incidence (18).

The prevalence and the incidence of PD increase with age, the extension of the life expectancy joined with the demographic shift in the population could explain the increasing number of cases observed worldwide. On the other hand, in the last two decades it was possible to observe a progressive increase in the mean age at onset of the disease and this aspect is not fully explained by population ageing, but suggest that probably additional variables may play a crucial role. Reasonably, the increasing awareness of the neurodegenerative chronic diseases, the improvement in the time and accuracy of diagnosis could be related with these cases. In fact early physical symptoms that in the past were related with ageing or missed by older patients now are more referred to the medical corps (19). This situation could also be explained by a reduction of cases at precocious incidence. The last twenty years have seen significant improvements in the medical management of risks factors of PD and, at the same time, the increasing consume of non-steroidal anti-inflammatory drugs may have contributed to attenuate the pathophysiologic role of neuroinflammation (20, 21). Despite the importance that pesticide and other toxic-compounds have on the precocious onset of the pathology, at our

days, thanks the progress and the attention also in this aspect of modern life, there is not a significant difference in the incidence of PD in people living in rural environment than people living in towns (22).

The etiology of PD remains to be fully understood, but the most plausible theories propose an environmental or genetic origin, or most probably a combination of both (23, 24).

Numerous processes are involved in the onset of the pathology like oxidative stress, mitochondrial dysfunction, inflammation, excitotoxicity and protein aggregation. In PD, α-synuclein protein (α-syn), the principal component of LBs inclusions, plays a key role in the pathogenesis of this disorder. The exact biological function of the protein and the mechanism by which mutations in this gene lead to neurons loss are still not clear, although it has been observed that an excess of α-syn depositions can cause dopaminergic neurons loss (25). On the other hand, oxidative damage induced by reactive oxygen species (ROS) participates in the progression of dopaminergic neurons degeneration. In particular, the metabolism of dopamine (DA) might be responsible for the high basal levels of oxidative stress in the SN; indeed, dopamine oxidation leads to the formation of neurotoxic species (26).

Currently therapy for PD is the administration of L-Dopamine (L-DOPA), a precursor of DA; but with the progression of the disease is necessary a higher dose to supply the endogenous deficiency of the neurotransmitter. Unfortunately this treatment is only palliative and symptomatic as it could not arrest the progression of the disease, but it can only supply the lack of DA. In the beginning of the therapy the drug administration presents good results because motor symptoms can be well managed, but with the massive loss of dopaminergic neurons also the pharmacological treatment loses its efficacy (27). Last but not least L-DOPA presents several side effects such as dyskinesia and motor fluctuation due to the variation of DA in CNS. This mechanism could act a dramatic plasticity’s reduction of dopaminergic

structures involving most of STR (28). Before that a symptomatic treatment was available, the progression of motor symptoms leads up to death in ten years from diagnosis. However expectancy of life strictly depends from the age of patients when the pathology arises.

1.1.2.TIPICAL NEUROPATOLOGY OUTCOME

PD is a multisystemic disease characterized by a slow progressive degeneration of dopaminergic neurons. The pathology presents different motor and non-motor symptoms. Usually the pathology is identified when first motor symptoms appear. Because of the compensative mechanisms of dopaminergic system, motor symptoms appear when more than 50% of dopaminergic neurons localized in SN are lost.

Four cardinal motor symptoms are the central features of PD: resting tremors, bradykinesia, rigidity and postural instability. Usually in the first appearance of these manifestations, it is possible to notice a side preference for the dominant one and gradually spread to the contralateral side, although the first side involved tends to remain the most severely affected. In this way many patients describe in the beginning micrographia, such as abnormally small and cramped, and other impairment in normal life task like fastening buttons. Specifically the four cardinal motor symptoms are those described below.

- **Resting Tremor**

Tremor may be the most visible sign of PD but rarely is the major cause of disability. This is a common initial symptom in 70-90% of patients is asymmetric rest tremor especially in younger one. This condition usually is distal and involves hands, such as thumb and the wrist with a “pill-rolling” movement. Resting tremor may also has a postural component and could show a leg tremor at rest (29).

- **Bradykinesia**

This symptom is the most disabling features of the disease and is reported from the 80-90% of patients. It consists in slowness in movement

and it contributes to the inability to arise from a chair, turn in the bed or get out of a car. Akinesia is the extreme manifestation of bradykinesia, and is define as the incapacity to start a movement (18). From this point of view appears clearly why this symptom compromises so deeply the patient's quality of life.

- **Rigidity**

90% of patients present this symptom that consists in resistance to the passive movement occurring both in flexor and extensor muscles during the entire range of motion. Usually it involves the distal part of limbs and could be continuous or intermittent.

- **Postural Instability**

Postural instability is a sign of more advanced PD, and also one of the most disabling motor features because predisposes to falls and consequently to injures. This symptom, although initially is responsive to treatment, often becomes resistant in short time (29). Probably this is because of the dramatic decrease of the number of dopaminergic neurons in the late stage of the pathology.

Epidemiologic and clinical data suggest that a wide variety of additional features (non-motor symptoms) may precede the traditional motor manifestation of PD by long periods (30). Current therapies act mainly on the dopaminergic system with the goal to improving motor symptoms. However, intrinsic non-motor symptoms of PD are increasingly recognized as being critical to identify and treat because of their impact on quality of life in PD, perhaps having an even greater impact than motor symptoms. Despite increasing evidence of them importance on the quality of life, studies have shown that there are several difficult in treatment of these issues (31). Although physicians may be aware that this features are common in PD, these gaps in treatment may be attributable the necessity for increased information about them, and clinical approaches for their assessment and management in the context of PD as a whole. The most common non-motor symptoms described are autonomic dysfunction, cognitive abnormalities,

sleep and mood disorders, pain and sensory disorders. In addition to them there are others that are secondary to pharmacotherapy treatment such as impulse control disorders and psychosis (32). Because PD is primarily recognized as a movement disorder, the natural focus on motor symptoms often leads to undervalue non motor symptoms by patients and clinicians. The importance of recognizing these features is highlighted by the expanding evidence that they cause significant morbidity for patients with PD, perhaps equal to the morbidity caused by the motor symptoms, and this is particularly true in patients with advanced disease (33).

1.1.3.ORIGIN AND RISK FACTORS

PD is a multifactorial and multisystemic progressive neurodegenerative disorder. The cardinal features of the disease are a result of the degeneration of the dopaminergic nigro-striatal pathway originating in the SNpc which terminals projects to STR. SNpc and STR, which is constituted by putamen and corpus caudate, are part of a complex neuronal system that include cortex, basal ganglia and thalamus that are responsible of the normal motor functions. While neurons of this region are preferentially lost in PD, they are not the only ones involved in the progression of the disease. Degeneration in other areas could contribute in many of the non-motor symptoms of PD, such as depression, dementia, and autonomic dysfunction (34). In addition to neuronal loss, surviving neurons contain LBs, which are cytoplasmic, eosinophilic inclusions always present in PD and predominantly composed by a protein called α-syn (2). Because of the natural ageing of the brain, is usual to find LBs inclusions in the brain of people that are not diagnosed with PD. It has been suggested that LBs as an incidental finding, are a feature of normal ageing without any additional nigral cell loss (34). In this way it is possible to conclude that the formation of LBs plays an important role in the progression of the pathology, however the compensatory mechanisms responsible of their elimination play a more important part. At the same time is important to underlie that these inclusions

are present in both, sporadic and familial, forms of PD (25). Current treatments address the dopaminergic deficit, providing symptomatic benefit to many of the motor deficits. However, there are many symptoms that do not respond to dopaminergic therapy. In addition, there are no proven interventions to slow down the underlying degenerative process. The search for such neuroprotective treatments is a critical goal of PD research. In order to find neuroprotective strategies, a clear understanding of the mechanism of neuron death in PD is needed. Early pathways are not directly linked to the execution of cell death. In contrast, distal effectors are intimately involved in the death process, and their recruitment directly regulates the likelihood of cell death (35) (Fig. 2).

Most PD cases are of sporadic origin, but about 15% are associated with genetic causes. From the various loci associated with PD, named PARKs, six proteins have been identified so far. They are linked to either autosomal dominant (α-synuclein, UCHL1, and LRRK2/dardarin) or autosomal recessive transmission (parkin, DJ-1, and PINK-1), that is more severe and characterized by an early onset (35). Among the proteins responsible for the familial forms of PD, α-syn has received particular attention, not only because it was the first gene product implicated in familial forms of the disease but also because it is the major fibrillar protein of the LBs. Even if its function is far from being completely elucidated, this protein seems to play a major role in cell death processes (36).

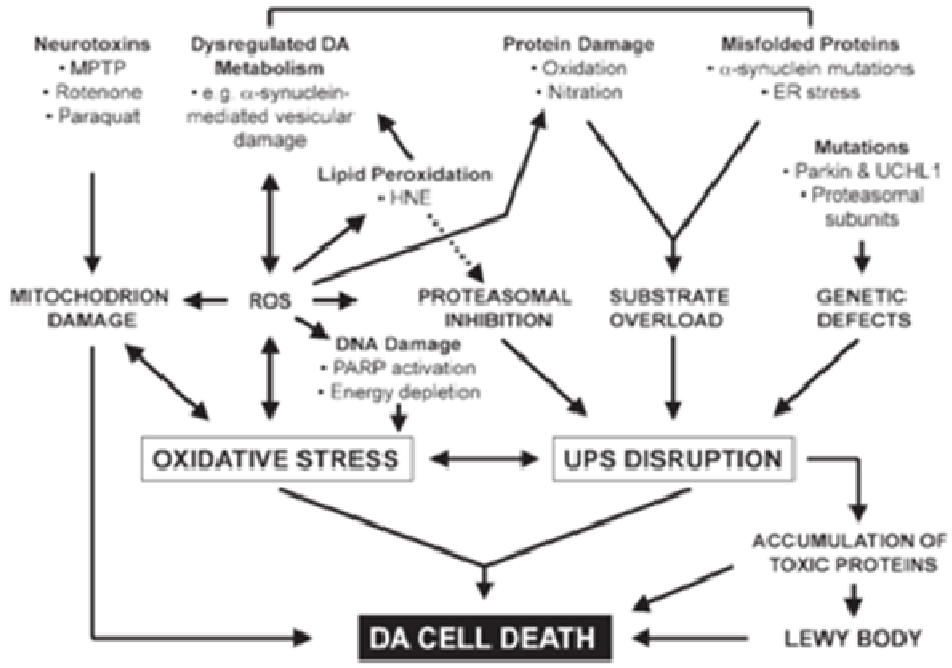


Figure 2. Cascade of events leads to dopaminergic cell death in PD. Various pathogenetic factors interconnect with each other in dynamic network that underlie PD pathogenesis.

PD pathogenesis is a complex and multifactorial process in which both genetic features and environmental stressors converge and compromise neuron viability by affecting the cellular systems dedicated to the maintenance of homeostasis: protein quality control systems and mitochondria (37).

Three major dysfunctions are observed in PD, oxidative stress, mitochondrial dysfunction and excitotoxicity, all of them associated with cell death. Also neuroinflammation plays a crucial role in the evolution of the disease. In fact markers, such as activated microglia and increased levels of circulating proinflammatory cytokines have been observed in patients' brains. Despite the fact that inflammatory process could contribute to neurons lost, the link between anti-inflammatory drugs and PD in humans remains uncertain. Some studies have shown how nonsteroidal anti-inflammatory drugs (NSAIDs) exerting neuroprotective effects in PD animal models (38).

Oxidative damage and mitochondrial dysfunction contribute to the cascade of events leading to the degeneration of dopaminergic neurons. Others neurodegenerative diseases are associated with oxidative stress, suggesting that this mechanism would be a common way to contribute to neuronal damage. Oxidative stress defines disequilibrium between the levels of ROS produced and the ability of biological systems to detoxify the reactive intermediates. All organisms have developed adaptive responses to oxidative stress that result in an increased production of defensive enzymes, molecular chaperones and antioxidant molecules (39). ROS can be generated through several pathways such as direct interactions between redox-active metals and oxygen species, reactions, or by indirect pathways involving the activation of enzymes like nitric oxide synthase (NOS) or nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (40). Superoxide anion radical (O_2^{2-}), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) are examples of ROS. O_2^{2-} is produced predominantly by mitochondrial complex I and III of the electron transport chain (ETC) and is highly reactive. It can cross the inner mitochondrial membrane where it can be reduced to H_2O_2. It can also be generated in peroxisomes which contain catalase that allow H_2O_2 to be converted in water, preventing its accumulation. However when peroxisomes are damaged, peroxide is released to the cytosol where it contributes to oxidative stress (41). The extensive production of ROS in the brain may provide an explanation for the magnitude of the role that these reactive molecules play in PD. The brain consumes about 20% of the oxygen supply of the body, and a significant portion of that oxygen is converted to ROS (42). Numerous evidences suggest that a significant contributor to dopaminergic neuronal loss in PD brain are ROS, which result from dopamine metabolism, but also low glutathione (GSH) levels, and high levels of iron and calcium in the SNpc (43). Additionally, the brain contains high concentrations of polyunsaturated fatty acids, which under oxidative stress conditions; result in lipid peroxidation and the generation of toxic products.

GSH is a tripeptide consisting of glutamate, cysteine and glycine, with the reactive thiol group of its cysteine residue serving as an effective antioxidant. The peptide is synthesized in the cytoplasm but has to be transported to the mitochondria, where it functions as an antioxidant molecule (44). GSH, with the help of enzymes glutathione peroxidase (GPx) and reductase (GR), forms detoxification machinery against these oxidative species. GSH levels are finely regulated in healthy neurons, and alterations from the physiological basal levels can induce cell death. The depletion during PD precedes mitochondrial damage and DA loss and the degree of its loss has been observed to correlate with disease severity (45) (Fig. 3).

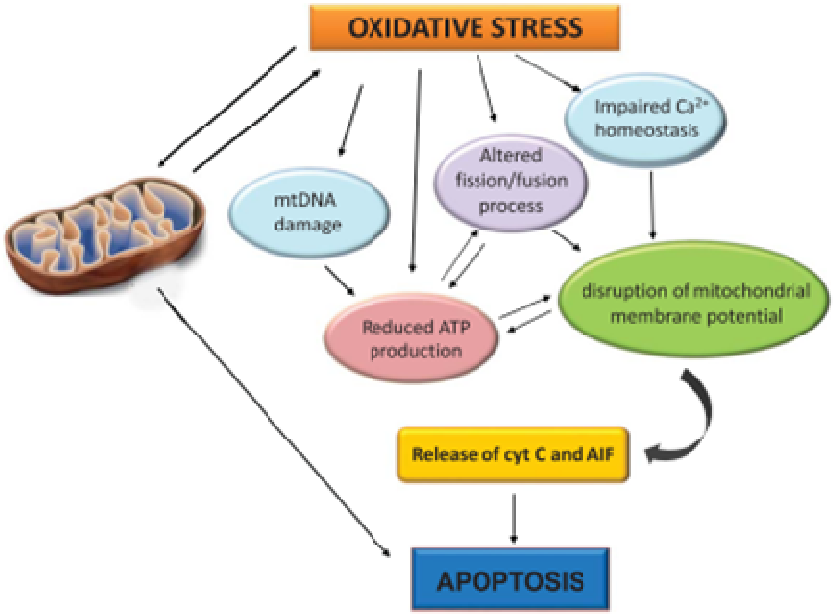


Figure 3. Oxidative stress and Apoptosis. Chemically reactive molecules containing oxygen are produced mainly in mitochondria as a consequence of the basal metabolic activity. Oxidatively damaged mitochondria and mutated mtDNA-encoded defective protein subunits will impair ECT, which results in less ATP production and more ROS outbreak. Increased ROS production promotes accumulation of mitochondrial Ca^{2+}, mitochondrial fragmentation, mtDNA damage, disruption of mitochondrial membrane potential, and release of cytocrome C (Cyt C). Mitochondrial function imbalance elicits a vicious cycle of ROS damage that ultimately triggers apoptosis.

During PD, there is a selective inhibition of mitochondrial complex I (CI) resulting in mitochondrial dysfunction (46). PD toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone act on dopaminergic neurons via selective CI inhibition. This complex is considered to be one of the most severely affected, in CTE, by age related oxidative stress, resulting in mitochondrial dysfunction (47) (Fig.4).

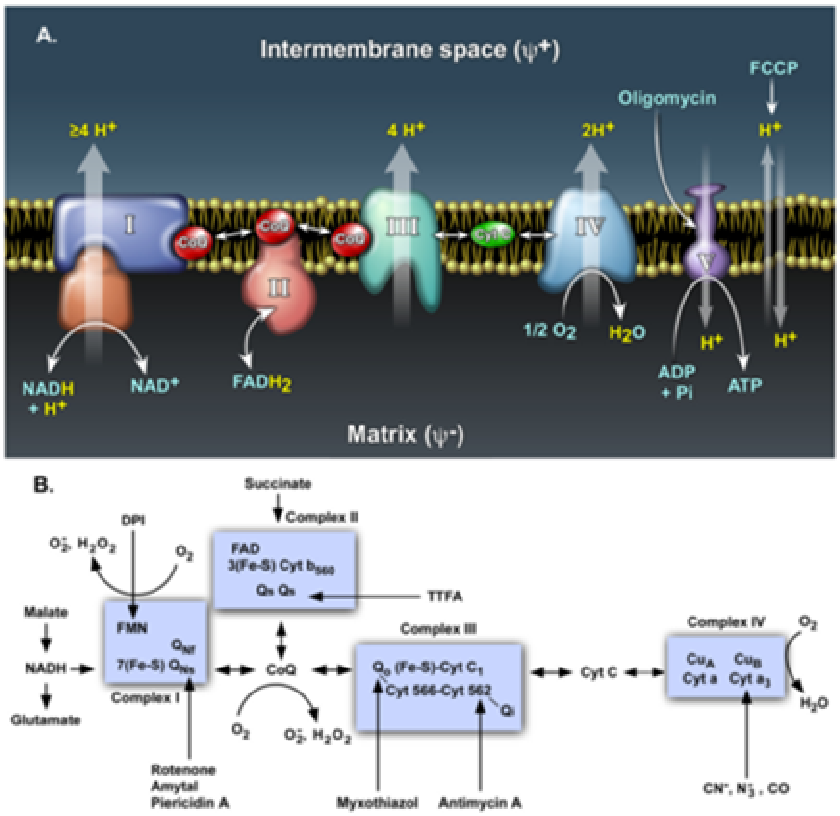


Figure 4. Oxidative phosphorylation and the mitochondrial electron transport chain. A: Oxidative phosphorylation: the membrane topology of mitochondrial complexes, the sites of proton translocation and the targets of agents that affect the transmembrane proton gradient. **B**: The mitochondrial electron transport chain: the sites of ROS generation and the sites of action of commonly used respiratory inhibitors.

It is therefore evident that neurodegeneration in PD involves an interaction between GSH depletion, oxidative/nitrosative stress, mitochondrial dysfunction, proteasome inhibition and protein aggregation. But the precise relationship among these pathways has not been completely defined. Because PD pathology simultaneously involves more than one

process, it would be interesting to evaluate the synergistic effect of simultaneously occurring processes. It is also possible that the presence of mutated α-syn protein or proteasome inhibition may impact on mitochondrial function and in combination result in alterations in GSH metabolism and by enhanced mitochondrial dysfunction in PD (48).

Another important mechanism involved in the pathogenesis of PD is excitoxicity, a pathological process through which neurons are damaged and killed after excessive stimulation of glutamatergic receptors by glutamate, the main excitatory neurotransmitter in the CNS. This phenomenon is involved in several pathological conditions affecting the CNS such as stroke, epilepsy and AD. Excitotoxicity is due to intracellular processes, such as calcium overload and bioenergetics changes, which increase the oxidative burden and activate apoptosis. Within the basal ganglia other neurotransmitter systems, such as GABAergic, cholinergic and glutamatergic, are present together with the dopaminergic system. In PD, the altered neurotransmission observed within the basal ganglia affects the glutamatergic system, thereby suggesting a critical involvement of glutamate-mediated excitotoxicity in the pathogenesis as well as in the progression of the neurodegenerative process. Glutamate-mediated excitotoxicity may be involved in a lethal vicious cycle, which critically contributes to the exacerbation of nigrostriatal degeneration in PD (38).

Not less important is neuroinflammation that has been identified in postmortem brain in the form of activated microglia pro-inflammatory cytokines in SN and STR and has been suggested as part of the pathophysiology of the disease (49). This inflammatory reaction is triggered by the presence of LBs beacuse α-syn protein is an activator of microglia. The magnitude of activation is dependent on the amount of the α-syn present. This can lead to the differentiation of microglia into different phenotypes, including antigen presenting or macrophage like forms. The first one correlates with lower levels of α-syn, also triggers activation of the adaptive immune system through CD4+ T-cells (50). Although higher levels

of α-syn are associated with a macrophage-like phenotype, this may not apply to all variations of α-syn protein, as specific mutations within the protein can trigger a pro-inflammatory phenotype, as opposed to a macrophagic one (51). Subsequent exacerbation can follow episodes of peripheral infection or a chronic inflammatory condition, causing neurotoxicity and the release of nitric oxide (NO) or ROS (52).

Finally is important to understand the role of environmental toxins in the disease's pathogenesis. Toxins could be inhibitors of CI, they could trigger the dopaminergic neurons death, or they could modify the normal mitochondrial physiology. Neurotoxins are frequently used to induce experimental models of PD in vitro or in vivo. Following three examples:

- **MPTP**, is a high lipophilic molecule able to pass the blood brain barrier (BBB). MPTP is oxidized by monoammine oxidase B (MAO-B) in its active metabolite, the toxin MPP^+. This metabolite is much more polar so it couldn't enter in neuronal cell but it need membrane transporter for dopamine (DAT). In this way MPP^+ is selectively concentered in dopaminergic neurons. In the cell the active metabolite is found preferentially in mitochondria where is able to interact with proteins of CI.

- **ROTENONE** is a common pesticide, inhibitor of CI. It is a lipophilic molecule so is able to pass BBB and is not selective for dopaminergic neurons. Rotenone promotes α-syn aggregation and LBs deposition (53).

- **PARAQUAT** a powerful weed killer able to induce inhibition of complex I and III and to promote α-syn aggregation. This molecule is not able to pass the BBB but its structure is very close to active metabolite of MPTP, the MPP^+, so is able to use the same membrane transporter (54).

1.1.4.CELL DEATH MECHANISMS IN PD

Cells respond to stress in different ways, they can activate pathways that promote survival or induce programmed cell death to eliminate damaged cells. The initial response to a stressful stimulus is regulated towards helping the cell to defend against it and recovering from the insult. However, if the noxious stimulus is unresolved, cells activate death signaling pathways. Survival critically depends on the ability to give an appropriate response towards environmental or intracellular stress stimuli. The adaptive capacity of a cell definitely determines its fate. Therefore, depending on the level and mode of stress, different defense mechanisms and pro-survival strategies are mounted; however, if these are unsuccessful, then the cell death programs are activated to eliminate these damaged cells from the organism. The mechanism, by which a cell dies, that is, apoptosis, necrosis, or autophagic cell death, often depends on its ability to survive in those conditions (55).

Differently from other cells, neurons have a long life because of them role in the neuronal system. Initially it is possible to see an overproduction of neurons, this condition is important because allows the system to reach the correct number of cells to build complicated neuronal circuits. After an injury that would be caused by a disease, or ageing or a trauma condition, neurons die, never be substitute and neuronal connectivity is lost.

Programmed cell death, apoptosis, is a physiological process that occurs naturally during life in which intrinsic molecular programs are activated to cause cell death. Inappropriate apoptosis control is implicated in many human diseases including neurodegenerative disorders: in these cases the apoptotic rate is increased and the consequent tissue damage may be evident. On the contrary, when apoptosis is reduced, cells that should be eliminated may persist and reproduce, like in tumors. Apoptosis is considered as the dominant mechanism for neurodegeneration in PD (56). It takes place rapidly and is characterized by a series of distinct morphological

and biochemical alterations such as condensation and fragmentation of nuclear chromatin, condensation of cytoplasmic organelles, and dilatation of the endoplasmic reticulum (ER) (57). There are two main apoptotic pathways, extrinsic and intrinsic. Although each pathway employs a different set of initiator caspases, both share the same executioner caspases. Caspases family includes 14 aspartic acid-specific cysteine proteases involved either in inflammation or apoptosis. They are synthesized as inactive zymogens called procaspases that require cleavage and dimerization for activation. Executioner caspases are responsible for most of the morphological and biochemical transformations observed in apoptotic cells. In the case of the extrinsic pathway, apoptosis is triggered by activation of
death receptor, members of the Tumor Necrosis Factor receptor (TNFR) family by their ligands, event that leads to the recruitment of procaspase-8 (58).

The intrinsic pathway is also known as mitochondrial pathway depending on the release of apoptotic factors from mitochondria (59). This pathway may play a major role in triggering apoptosis in PD (58). The intrinsic pathway is activated by cellular stresses like growth factor deprivation, cytoskeletal disruption, DNA damage, ROS production, and accumulation of unfolded proteins. All these changes influence the inner mitochondrial membrane with the opening of the mitochondrial permeability pores, loss of the mitochondrial transmembrane potential, and release of pro-apoptotic proteins from the intermembrane space into the cytosol. The central phenomenon in this pathway is the release by mitochondria of cytochrome C (cyt C) which induces polymerization of cytosolic Apaf-1, adaptor protein of the intrinsic pathway that it is required for activation caspase-9 (60). This complex then binds procaspase-9 to activate it in the active form caspase-9, which, together with Apaf-1 forms the apoptosome complex. Finally, the apoptosome complex plays a role in activation of caspase-3 (58). This mechanism is highly regulated by proteins of the Bcl-2

family, comprising members that have either anti-apoptosis (such as Bcl-2 and Bcl-xL) or pro-apoptosis (such as Bax and Bak) effects (61). Structurally, all these proteins share some degree of similarity and can have up to four Bcl-2-homology domains (BH_1 and BH_4). In addition to the Bcl-2 members that contain multiple BH domains, there are molecules that share sequence homology only with the BH_3 domain, which induce cell death by playing a crucial role in activating Bax, either by directly facilitating Bax oligomerization and translocation to the mitochondria or by inactivating anti-apoptosis proteins (62).

Crosstalk between the extrinsic and intrinsic pathways occurs through caspase-8-mediated cleavage of the domain of Bcl-2 protein that interacts with death agonist. This is able to translocate to mitochondria where it can cooperate with pro-apoptotic Bax and Bak in determining the permeabilization of the mitochondrial membrane (62). The changes of mitochondrial membrane integrity result in the decrease of the mitochondrial transmembrane potential and in the release of several pro-apoptotic proteins (63). This release in turn causes disturbances of mitochondrial transmembrane potential and the alteration of cell's biochemical homeostasis since ATP synthesis is prevented while NADH, NADPH, and glutathione are oxidized and ROS excess is produced (64).

The extrinsic and intrinsic pathways both activate the executioner caspases that modulate the beginning of the "execution phase" of apoptosis. Caspase-3, -6, and -7 function as apoptotic effectors, cleaving different cellular substrates and activating cytoplasmic endonucleases and proteases that degrade the nuclear material and cytoskeletal proteins respectively (65) (Fig 5).

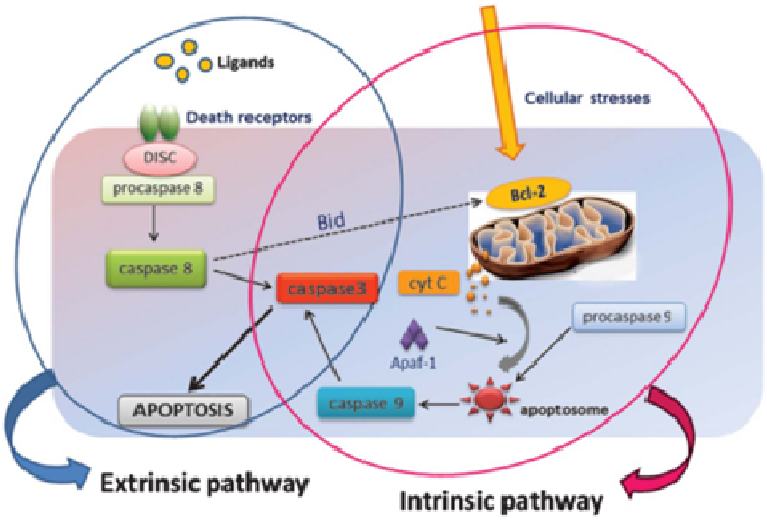


Figure 5. Extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is activated by death receptor activation. The intrinsic pathway is activated by cellular stresses which result in the perturbation of mitochondria and the release of pro-apoptotic factors such as cyt C that, once released, binds the adapter protein Apaf1 to form the apoptosome complex leading to the activation of caspase-9 and the downstream elements of the caspase cascade. Crosstalk between the two pathways occurs through caspase 8-mediated cleavage of the Bcl-2 protein Bid.

Cell homeostasis is disrupted by intracellular accumulation of aberrant proteins, inactive enzymes and damaged organelles. Cell response involves primarily two major systems for whole protein degradation: the proteasome system and autophagy, the latter one has the ability to degrade entire organelles.

Proteasome inhibition is another key event affecting neuronal death in PD. The tumor suppressor gene p53 is a sequence-specific transcription factor that increases dramatically in response to a variety of cellular stresses such as DNA damage or oxidative stress. The p53 is activated by multiple post-translational modifications with subsequent stabilization. Once it is stabilized, p53 enters the nucleus and regulates the transcription of several genes that promote cell death (66). The p53 is also known to be able to cause cell death by directly inducing mitochondrial permeability and apoptosis, independently of the transcriptional up-regulation of pro-apoptotic genes and may interact with numerous Bcl-2 family members (35). The regulation of p53 levels in the cell is complex and not completely

understood. However, the ubiquitin proteasome system is known to play an important role in maintaining low levels of p53 in the cell (67). Accordingly, proteasomal dysfunction in PD could lead to increase levels of p53 that contributes to the degeneration of dopaminergic cells (Fig. 6). Dopaminergic cell death is accompanied by a non-transcriptional increase in phosphorylated and then activated p53, and p53 inhibition prevents cell death in these models. Phosphorylated p53 accumulates in the SN of mice treated with proteasome inhibitor as well as in patients with sporadic PD. These data suggest that p53 signaling plays a key role in cell death associated with proteasome inhibition and possibly in PD itself. During PD, SN neurons accumulate proteins, leading to formation of intraneuronal deposits, LBs (68). α-syn, the major component of LBs, is a presynaptic protein with a probable role in synaptic function. Under normal physiological conditions α-syn is lipid associated but tends to aggregate in the lipid free-state into higher molecular weight oligomers (69). Formation of these aggregates has been directly linked to neurodegeneration in PD.

Glutamate receptors over-activation is another important apoptotic stimulus for neurons. Accumulation of extracellular glutamate and increased stimulation of glutamatergic neurons spreads ROS production inducing oxidative stress. Stimulation of these receptors involves calcium homeostasis dysfunction, caspases activation, increase in cytotoxic transcription factors and free radicals. Excitotoxicity may occur in acute neurodegenerative conditions as well as in neurodegenerative chronic diseases (60).

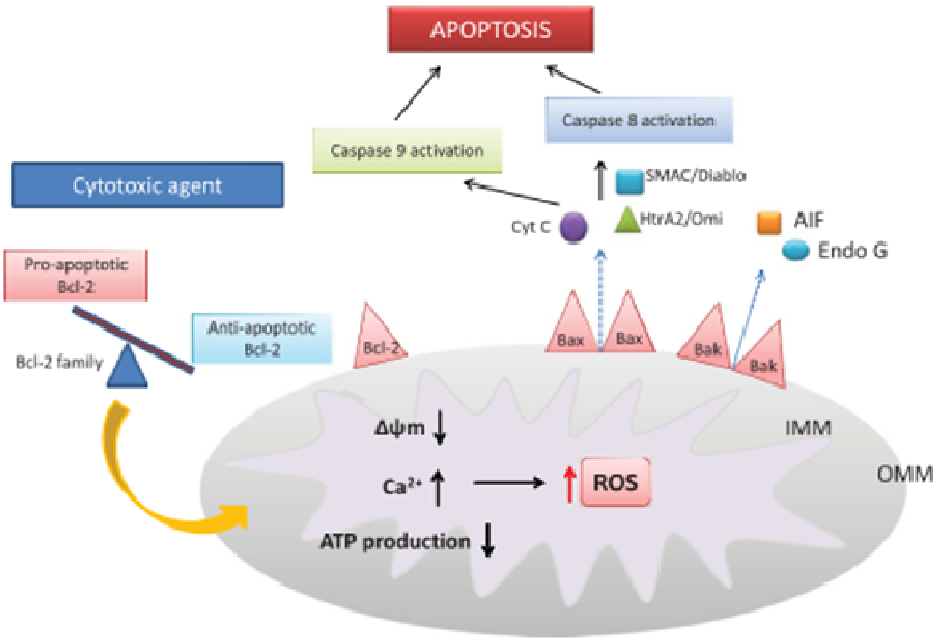


Figure 6. Mitochondrial functions in the regulation of apoptosis. A major key point in the regulation of apoptosis is the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family members. Upon receiving an activating signal, the pro-apoptotic members directly engage and activate the pro-apoptotic effectors Bax and Bak making them homodimerize and causing their translocation to the outer mitochondrial membrane (OMM). Pro-survival Bcl-2 proteins constitute a checkpoint for Bak/Bax homodimerization and in turn they can be repressed by sensor Bcl-2 proteins. Once inserted into the OMM, Bax/Bak homo-oligomers lead to pores formation and MOMP causing the release of several pro-apoptotic proteins. The release of mitochondrial factors in turn causes alteration of the biochemical homeostasis of the cell since ATP synthesisis prevented, NADH, NADPH, and GSH are oxidized and excess of ROS is produced.

Finally, another key mechanism of cell death in PD is autophagy. Autophagy is a process induced by a change in environmental conditions, such as nutrient deprivation, oxidative stress and ultraviolet radiation. This mechanism is also been associated with normal procedures like development, differentiation and defense. Sometimes, in spite of offering protection, it may also contribute to cell damage (70). There are three arms of the autophagy pathways: macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy. The first one becomes activated under stress conditions such as nutrient deprivations or toxins and it is

characterized by the formation of a autophagosome that merges with lysosomes (Fig. 7).

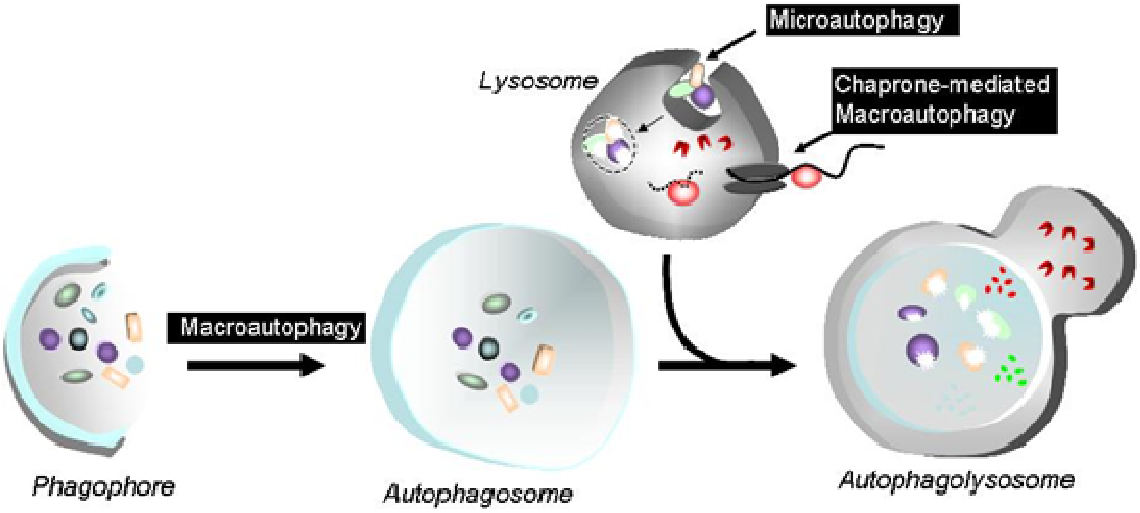


Figure 7. Autophagy pathways.

CMA involves directly lysosomal targeting and degradation of soluble, cytoplasmatic proteins that present a specific target sequence. This mechanism plays a key role in α-syn turnover and probably its pathogenic effects. Finally in the latter, the lysosome surrounds a targeted protein or organelle. All these processes maintain intracellular balance and can occur in every living cell. When cell homeostatic mechanism fail to comply, death is inevitable. This leads to the induction of mitosis of neighbouring cells in order to maintain tissue integrity and function. In the adult brain tissue, this process of replenishment cannot occur, since neurons cannot divide. Neurons have to adjust all their intracellular processes in order to survive for decades. Thus, proper autophagy regulation is essential for neuronal survival and its dysfunction has been entailed in neurodegenerative disease (71).

The α-syn recycling is performed by the proteasome system, CMA and macroautophagy. The proteasome degrades only soluble forms of the protein. A percentage of intracellular α-syn modifies the CMA pathway,

having weak affinity for the CMA receptor on the lysosomal membrane. Instead of entering for degradation, they accumulate in the cytoplasm (72).

Finally, the PD-related proteins PINK1 and Parkin operate as central components of autophagy. Overall, these studies highlight an emerging concept where perturbations in the function of autophagy and the lysosomal pathway may contribute to the development of neurodegenerative conditions as part of the etiology of the disease, affecting the global maintenance of proteostasis and catabolic processes involved in the clearance of damaged organelles (72).

1.1.5. ANIMAL MODELS

Animal models are essential research tools not only to explore the underlying pathology and molecular mechanisms of disorders but also to evaluate the potential efficacy of the interventions; and to provide an initial estimate of the safety margin and human dosing parameters of a drug candidate. There are numerous limitations to the use of such models, not the least of which is the inherent challenge associated with attempting to model complex and still poorly understood human disorders in a lower species. This task is particularly difficult for CNS disorders due to the poverty of information about the genetic and epigenetic origins and molecular mechanisms responsible for these disorders, the heterogeneous nature of many of these conditions and the subjective and sometimes contradictory endpoints that are used to describe their symptoms and severity (73).

The two categories of PD models are toxin-based models and transgenic mice. Neurotoxic models of PD include that produced by the toxin 6-hydroxydopamine (6-OHDA) into dopaminergic cell or terminal regions, most commonly used in rats, but also in mice and marmosets. The second one is by MPTP, which is currently almost exclusively used in mice and in a variety of non-human primates. However the administration of MPTP to various animals (monkeys, mice, cats, rats, guinea pigs, dogs, sheep, and

even frogs and goldfish) has been shown to also cause parkinsonian like motor disturbances. This neurotixin was inadvertently produced during the synthesis of an analog of Demerol for recreational use. Ingestion of the compound by young drug abusers resulted in the production of motor symptoms that were indistinguishable from PD. MPTP has subsequently been shown to be converted to MPP+ in the brain which is preferentially taken up into dopaminergic neurons by the DAT. Once inside the neuron, it blocks the ECT in the mitochondria, decreasing cellular ATP levels and leading to the formation of toxic ROS. It should be noted that MPTP-treated mice also exhibit preferential loss of cells in the SNpc and they show motor impairments (2). Other neurotoxins are rotenone, paraquat and maneb, isoquinoline derivatives, and methamphetamine. Although it is important to cite the attempts to produce new models with the latter toxins, so far, none has succeeded in generating new and reliable therapies (74, 75) (Table I).

Table I. Differences in toxins induced animal models

Toxin	Time to greatest DA cell loss	Striatal loss of dopamine	Advantages	Disadvantages
6-OHDA in SN	42h	Dose-dependent loss of DA innervation	1. Full DA depletion of nigrostriatal pathway 2. Mimics late-stage PD 3. Test therapeutic strategies	1. Not progressive 2. like axotomy 3. No extra-nigral pathology
6-OHDA in STR	16 weeks	Circumscribed loss of TH immunoreactvity at injection site	1. Progressive DA cell loss 2. Produced incomplete lesions that mimic PD	1. Strong striatal glial reaction 2. No inclusions 3. No extra-nigral
MPTP	24 h	Dorsal striatum with sparing of nucleus accumbens	1. Inhibits complex I activity 2. Striatal TH loss	1. Not progressive 2. No inclusions
Paraquat and Maneb	7 days	Little or no measurable changes in striatal DA innervation	1. Combination of paraquat and maneb is more effective for DA depletion	1. Inconsistent results on DA loss 2. No inclusions 3. No extra-nigral
Rotenone	36 days or more	Dose-dependent loss of TH in dorsal striatum with sparting of nucleus accumbens	1. Inhibits CI activity 2. Progressive DA cell loss 3. α-syn inclusions in DA neurons 4. I.p. administration shows extra-nigral pathology	1. Large variations in animal sensitivity 2. Motor deficits have yet to be demostrated

There are relatively rare forms of PD that have been linked to genetic mutations. The two autosomal dominant genes are α-syn and leucine rich repeat kinase 2 (LRRK2). The formarmation of LBs is a hallmark pathologic feature of PD and point mutations or duplications are sufficient to cause PD. Several α-syn transgenic lines have been created and their phenotype heavily depends on the choice of promoter. None of the models accurately represent PD, while there are some dopamine-responsive functional abnormalities; there is no progressive loss of dopaminergic neurons. Only transgenic mice with the prion promoter (mPrP) exhibit the full range of α-syn pathology that is observed in humans (76). LRRK2 is a large multidomain containing protein that is localized to membrane structures and mutations linked to PD are concentrated in the GTPase and kinase domains (77). Transgenic LRRK2 mice have abnormalities in the nigrostriatal system and behavioral deficits that are dopamine-responsive. However, they display a very mild phenotype with minimal evidence of neurodegeneration and are clearly not robust models of PD. There are several autosomal recessive genes that have been linked to PD and the best characterized of these are parkin, DJ-1 and PINK1. Knockout mice have been created for each of these genes but none of them exhibit nigrostriatal pathology, however they could be useful for the exploration of early, pre-neurodegenerative changes that occur in PD (75).

- 6-OHDA INDUCED LESION

6-OHDA is the neurotoxin par excellence to model PD in rats and is also being increasingly used with genetically modified mice. When administered systemically, 6-OHDA destroys sympathetic neuron nerve terminals in the peripheral nervous system. The toxin shares some structural similarities with dopamine and norepinephrine (Fig. 8), this is the reason because is preferentially transported into dopaminergic neurons by the DAT and in noradrenergic neurons by the norepinephrine transporters (NET).

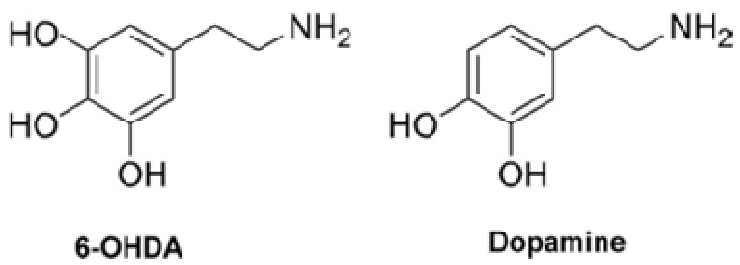


Figure 8. Comparison of the chemical structures of 6-hydroxydopamine and dopamine

Once in the neuron, 6-OHDA accumulates in the cytosol and undergoes prompt auto-oxidation, promoting a high rate of H_2O_2 formation and para-quinone; so the neurotoxin can inflict damage to the catecholaminergic pathways by a combined effect of ROS and quinones (Fig. 9) (78, 79). As an additional mechanism, 6-OHDA can accumulate in the mitochondria, where it inhibits CI activity. The lesion obtained with 6-OHDA is highly reproducible, which represents a considerable added value when new therapeutic strategies are to be investigated and clear neuroprotective effects must be demonstrated (80).

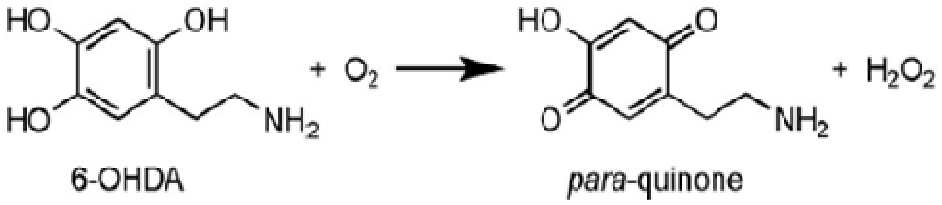


Figure 9. 6-OHDA oxidation

Like other parkinsonian neurotoxins, 6-OHDA can be administrated by systemic injection, but will not produce the desired nigrostriatal lesion. The toxin poorly crossed the BBB, to circumvent this problem 6-OHDA has to be injected stereotaxically into SN o STR to affect directly the nigrostriatal dopaminergic pathway. Supporting the relevance of oxidative stress in this model, it has been shown that mice over expressing superoxide dismutase and glutathione peroxidase are protected against 6-OHDA toxicity, as are rats treated with the antioxidant vitamin E (80). After an injection in SN, dopaminergic neurons start to die within the first 24h and show non-apoptotic morphology. Maximal reduction of striatal dopamine level is

reached within 3–4 days after lesion, and, in most studies, residual striatal dopamine content is less than 20% of controls (81).

On the other hand, when injected into the STR, 6-OHDA produces a massive anterograde degeneration of the nigrostriatal system which can last from 1–3 weeks after lesion, and the dying neurons exhibit a varied morphology. In addition to the lesion of the dopaminergic system, gliosis is also a prominent feature of the 6-OHDA model. Many data support the idea that the glial response in experimental models of PD, especially of microglia, exacerbates the degeneration of dopaminergic neurons (82).

Unilateral injection of 6-OHDA produces a typical asymmetric circling-motor behavior that depends on the importance of nigrostriatal lesion. This is possible because of the rapidity with which the toxin is metabolized. In this way, with a unilateral injection the healthy hemisphere could be used such as internal control for the lesioned one. This specific behavioral abnormality is most prominent after administration of drugs that stimulate dopaminergic receptors, such as apomorphine (rotation away from the lesion), or drugs that stimulate the release of dopamine, such as amphetamine (rotation toward the lesion), due to physiologic imbalance between the lesioned and the unlesioned STR (83). Quantification of this turning behavior has been used extensively to assess the antiparkinsonian potency of new drugs, transplantation, and gene therapies and to study the motor fluctuations in the chronic treatment with levodopa (53). Also important to remember is the instrumental role played by the unilateral 6-OHDA rat model in the identification of key neurotransmitter pathways governing the functional neuroanatomy of the basal ganglia. In conclusion, although this specific model may be more challenging to use than some others, the huge body of work based on its utilization represents a significant force for choosing it in a variety of investigations. In keeping with this, the unilateral 6-OHDA rodents' model has been and continues to be one of the most popular experimental models of PD when it comes to the preclinical testing of new symptomatic therapies and neuroprotective interventions (54).

1.2. STRATEGIES OF NEUROPROTECTION

At these days, the pharmacological treatment for neurodegenerative pathologies is only symptomatic for motor features. In PD, the depletion of DA is reestablished by the assumption of L-DOPA, but with the progression of the pathology, the number of neurons is continuously diminished and in this way the drug cannot be efficient because it cannot found its target. On the other hand, the transformation itself of L-DOPA in DA contributes to the increment of ROS and consequently to the progression of the neurodegenerative process. For all these reasons is always much more important try to find out a therapeutic alternative.

Neuroprotection can be classically defined as the consequence of any intervention that produces long lasting benefits by influencing etiology or pathogenesis through preventing disease's onset or clinical decline. This is defined as the capacity of any intervention to normalize the function of injured (but not dead) neurons. At the bottom of the strategy there is the implication that neurons may be dysfunctional but not irreversibly damaged and therefore capable of being restored to normal functions. All these concepts imply that the main actors in neuroprotection are the pathogenesis of PD (particularly cell death) and the clinical evolution. At present, neuroprotective strategies in PD are not aimed at treating the consequences of the disease, but to interfere with the basic pathogenic mechanisms of nigral cell death (84). However, PD is better defined as the final consequence of a degenerative process linked to a special vulnerability of cathecholaminergic cell groups and a failure of compensatory mechanisms associated with the effects of ageing and brain lesions. Furthermore, the preservation of normal neurons' functions can also lead to a better outcome and, consequently, it should be considered as an important target for any neuroprotective therapy. In keeping with this line of thinking, it is possible to lead another concept of neuroprotection such as an intervention able to delay PD clinical progression by directly or indirectly influencing disease

pathogenesis with a resultant reduction of cell death, cell damage and cell dysfunction. From a practical point of view, it implies that an improvement in general health, which can reduce the risk of vascular lesions, or in the ageing process, could exert a neuroprotective effect. Finally, it emphasizes the importance of gaining insight into the interaction between these new elements, PD pathogenesis and clinical expression since this could result in new therapeutic targets (85). Last but not least, some life style recommendations, such as the intake of hypocaloric and salt free diet with a high antioxidant content and the performance of regular physical and intellectual activities, might positively influence the final outcome by strengthening the compensatory mechanisms. All of these interventions have been shown to exert neuroprotective effects in experimental models by increasing the levels of trophic factors, by contributing to the correct functioning of the regenerative neural stem cells system (86). Generally a neuroprotection strategy can be summarized in three cardinal phases:

- **Primary** consists in the elimination of all risk factors
- **Secondary** is bind to the precocity of the diagnosis, when the pathology is still in an asymptomatic phase. This is at the bottom of an efficient strategy able to delay neuronal death.
- **Tertiary** consists in the slowing down pathology progression.

Many different mechanisms are involved in neurodegenerative disease such as excitotoxicity, ROS formation and propagation, and the activation of different cell death pathways. From the moment that PD is a multifactorial pathology, is extremely important that a new therapeutic approach has a large variety of target.

1.2.1.MULTIFUNCTIONAL PHYTOCHEMICALS

The theory of free radicals in the ageing process was first formulated in the 1950s by Harman. Following studies have indicated that oxidative damage constitutes a mechanism of injury found in many types of age-related diseases (87). As we age, antioxidant defenses become diminished, resulting in the increase of oxidative stress which is considered an important component in age-related diseases such as AD and PD (88). Unfortunately, the use of relatively safe antioxidant compounds found in the diet as a form of treatment in these disorders is attractive but limited by the difficulty in reaching an active concentration in the brain (89). Natural antioxidant molecules have been proposed as an alternative form of treatment for the prevention of these neurological pathologies. There are clear evidences that a diet rich in specific nutritional food groups like fruit, fish, and vegetables can reduce the incidence and prevalence of some of the main clinical features, such as neurodegenerative disorders, cardiovascular diseases, diabetes and cancer.

Brassica family is the largest and most widely consumed group of plants in Europe and all over the world. The family includes around 375 genera and about 3200 species characterized by different levels of nutrients. Because of their large and frequent consumption, they may become a significant source of nutrients and bioactive compounds in the daily diet. The beneficial effects of Brassica vegetables on human health have been linked to phytochemicals. They prevent oxidative stress, induce enzymes of detoxification, stimulate immune system, decrease the risk of cancers, inhibit malignant transformation and carcinogenic mutations, as well as, reduce proliferation of cancer cells. Brassica vegetables contain a lot of valuable metabolites, and a considerable source of antioxidants. Moreover, these vegetables are also rich in glucosinolates, which are unstable compounds and undergo degradation into biologically active indoles and isothiocyanates (ITCs) under the influence of enzyme presented in plant tissues,

myrosynase. These substances through the induction of enzymatic systems I and II phase of xenobiotics metabolism may affect the elimination or neutralization of carcinogenic and mutagenic factors, and consequently inhibit DNA methylation and cancer development (90).

1.2.2.GLUCOSINOLATES

Among the many varieties of vegetables, brassicaceae vegetables have received the most attention because of their unique constituents, glucosinolates. These components are abundant in edible parts and are regarded as most likely to maintain human health through continuous consumption. Glucosinolates are secondary metabolites found in Brassicaceae and related families, they present three different components: a β-thioglucose part, a sulfonated oxime, and a variable aglycone side chain derived from α-aminoacid. Glucosinolates, of which nearly 200 types having different substituents have been identified, are classifiable into three classes based on the structure of different amino acid precursors: aliphatic glucosinolates, indole glucosinolates, and aromatic glucosinolates (Fig. 10).

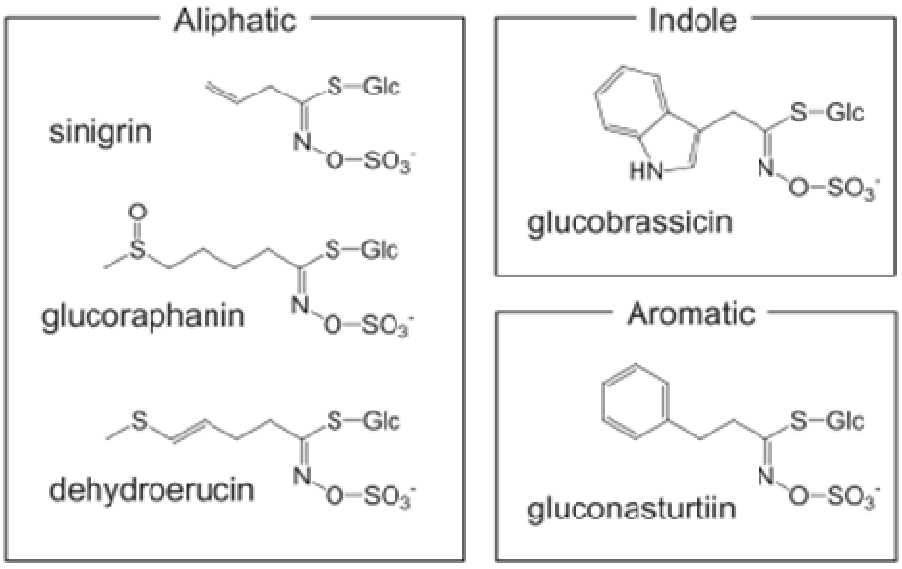


Figure 10. Glucosinolates found in Brassicaceae vegetables

The glucosinolates of each group are synthesized through a metabolic pathway that is independent and which shares a common set of enzymes

involved in the core structure formation of glucosinolates under genetic control (91). The composition and contents of glucosinolates are influenced by the genotype, climate and cultivation conditions including fertilization, harvest time and plant position (92).

Glucosinolates are not the bioactive compounds in cruciferous vegetables, but their hydrolysis products, ITCs are the activated structures. This is important because when plant tissue damage occurs by disruption, glucosinolates are hydrolyzed quickly upon a β-thioglucoside glucohydrolase enzyme called myrosinase, resulting in production of ITCs, thiocyanates, nitriles, goitrin and epithionitriles, depending upon pH and other conditions (93). When humans consume cruciferous vegetables the only sources of myrosinase activity are from plant endogenous enzymes and from intestinal microflora. The system, in which glucosinolate and myrosinase come into contact when tissue destruction occurs, is called “the glucosinolate-myrosinase system”. Presumably, glucosinolates are hydrolyzed only slightly under intact conditions, in which myrosinases are separated from the location of glucosinolates. Once tissues are mechanically damaged, glucosinolates are hydrolyzed intensively by myrosinases in a highly unstable aglycone intermediate. Although ITCs are main products from the myrosinase reaction, aglycones undergo rearrangement to form nitriles, depending on the structure of the intermediate, temperature and pH (94). Moreover, this glucosinolate-myrosinase system has been described as related to plant-insect and plant-pathogen interactions. Several studies have implicated glucosinolate degradation products in plant defense against insects, pathogens, and herbivores (95). Myrosinases are localized in myrosin cells, which are protein-rich idioblasts found mainly in the tissues of imbibed seeds. Myrosinase was localized in the epidermis and the vascular cambium of the radish and turnip taproots and the Japanese horseradish (wasabi) rhizome (96). This distribution of myrosinases, called a “double castle wall structure” is likely to be common among Brassicaceae vegetables. In the flower stalk of Arabidopsis, myrosinases are expressed

both in the phloem cells and the guard cells, but glucosinolates accumulate in the S-cells which are adjacent to the phloem cells. In fact, spatial separation of myrosinases from glucosinolates is the basis of the glucosinolate-myrosinase system. Myrosinase is destroyed by mild heating and cooking of vegetables, dramatically reducing isothiocyanate generation during chewing and digestion, however some isothiocyanate formation can occur in the gut due to myrosinase activity associated with endogenous microbes (97).

Glucosinolates are biosynthesized from amino acids. The three glucosinolate subtypes have their corresponding precursors: aliphatic glucosinolates are derived from alanine, leucine, isoleucine, valine, and methionine; indole glucosinolates and aromatic glucosinolates are derived respectively from tryptophan and phenylalanine or tyrosine. The glucosinolate biosynthetic pathways comprise three independent steps: the chain elongation stage, formation of a core glucosinolate structure, and secondary modification. The chain elongation also takes place in the biosynthesis of aromatic glucosinolates, but does not occur in the formation of indole glucosinolates.

Amino acids, including elongated ones, then undergo following step: the formation of core glucosinolate structure. Cytochromes P450 convert the amino acids to aldoximes, which are then oxidized to the activated forms by CYP83s. The activated forms are transformed to thiohydroximates via glutathione conjugation and the C-S lyase, and finally converted to the glucosinolate structure by the S-glucosyltransferases of the UGT74 family and the sulfotransferases SOTs. After the glucosinolate structure formation, the side chains are modified by oxygenation, hydroxylation, alkenylation, benzoylation, and methoxylation. The S-oxygenation of aliphatic glucosinolates is a common modification conducted by flavin monooxygenases FMOGS-OXs. The S-oxygenated aliphatic glucosinolates, such as glucoraphanin, are found in many Brassicaceae vegetables. Alkenyl

glucosinolates such as sinigrin are produced by 2-oxoglutarate-dependent dioxygenases AOPs from S-oxygenated glucosinolates (Fig. 11) (98).

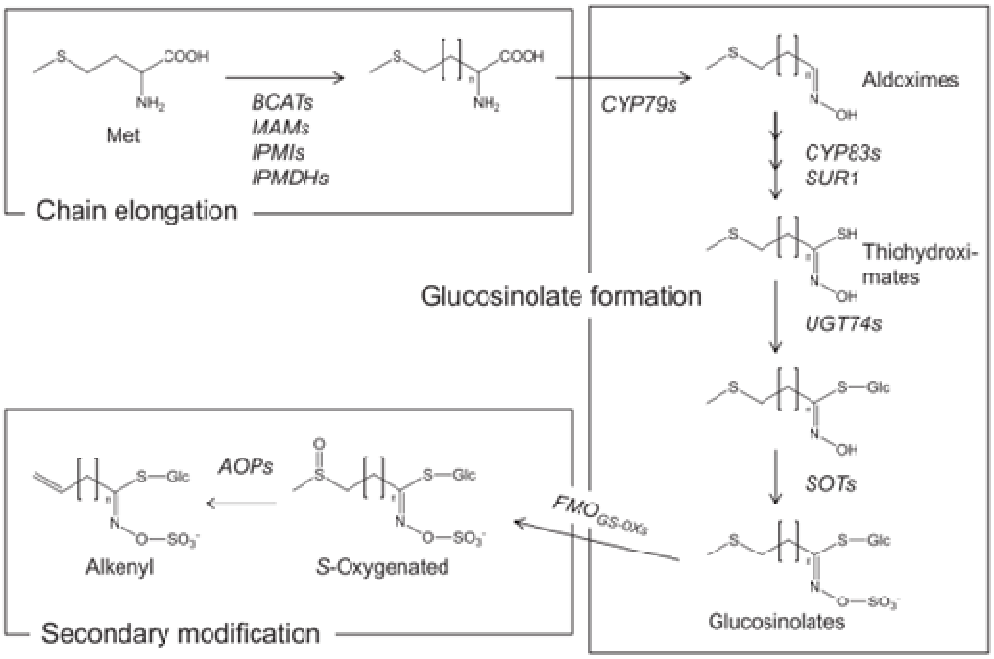


Figure 11. Schematic pathway of aliphatic glucosinolate biosynthesis. The pathway consists of chain elongation, glucosinoate formation, and secondary modification.

1.2.3.BIOLOGICAL ACTIVITY OF ISOTIOCYANATES

ITCs are a class of secondary metabolite responsible for the bitter taste and pungent smell of cruciferous vegetables such as broccoli, watercress, mustard and wasabi (98). Disruption of plant tissue results in the hydrolysis of inert glucosinolates by the enzyme myrosinase (Fig. 12).

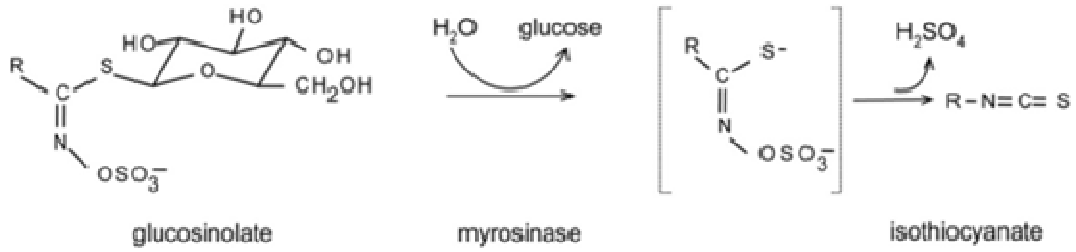


Figure 12. Myrosinase-dependent formation of ITCs.

These kinds of components have also attracted attention for the prevention and treatment of human disease. Humans obtain ITCs through the consumption of cruciferous vegetables. A large number of evidence indicates a positive association between increased consumption of cruciferous vegetables and decreased cancer risk. ITCs are reactive electrophiles that covalently modify proteins that will also be central to the effects of them in biological systems. These effects include triggering noxious responses, influencing carcinogen metabolism impairing tumor development and modifying inflammatory cytokine production. Accumulating evidences suggest that they exert their effects through a variety of signaling pathways involved in detoxification, inflammation, apoptosis, and cell cycle regulation. The central electrophilic carbon of ITCs (R-N=C=S) undergoes rapid addition reactions with biological nucleophiles, in particular, amines and thiols. ITCs react with amines to generate stable thiourea derivatives, whereas reaction with thiols generates labile dithiocarbamate adducts. Seminal studies by Drobnica and colleagues through the 1970's characterized the reactivity of a variety of ITCs with small molecules, peptides and proteins, and showed that they are able to react up to one

thousand times faster with thiol groups than with amino groups, rendering proteins with functional and structural cysteine residues particularly sensitive targets for modification (99). The tripeptide glutathione (γ-Glu-Cys-Gly) is an abundant physiological thiol, and catalysis by glutathione S-transferases ensures that a significant proportion of ITCs are initially conjugated to glutathione. These conjugates are rapidly effluxed from cells, and the removal of glutamate and glycine followed by acetylation generates a mercapturic acid (N-acetylcysteine isothiocyanate) that is excreted into urine. However, in all of these compounds the cysteine-isothiocyanate conjugate is able to dissociate back to the parent ITCs (100). This reversibility provides the opportunity for transport throughout the body, and the reaction of free ITCs or transfer reactions with more reactive targets (101). ITCs are particularly adept at inducing apoptosis. Their pro-apoptotic activity was first reported in the late 1990s and it provided the possibility of direct anti-cancer activity to complement the increased carcinogen detoxification displayed by these phytochemicals. Pro-apoptotic activity has often been associated with disruption of mitochondrial function, and in many cell lines cyt C release is observed quickly following ITCs exposure. Release of apoptotic mediators from mitochondria is regulated by the Bcl-2 family of pro- and anti-apoptotic proteins. A decline in levels of the anti-apoptotic proteins, as well as upregulation of the pro-apoptotic proteins have been reported in different models of isothiocyanate-induced apoptosis, suggesting that ITCs act by transcriptional or post-translational regulation of these proteins (99). Many structural and regulatory proteins have critical cysteine residues that are susceptible to oxidation. As a consequence, cells require a network of antioxidants to maintain a reducing environment. The antioxidant-response element (ARE) is a cis-acting DNA element essential for transcriptional activation of phase-II genes such as those encoding UDP-glucuronosyl transferase and glutathione-S-transferase and the genes for antioxidants such as NADPH: quinone reductase and heme oxygenase-1 (HO-1). The activation of ARE is dependent on the translocation of nuclear factor E2–related factor 2 (Nrf2) into the nucleus through its release from kelch-like

ECH-associated protein 1 (Keap1), the cytoplasmic repressor of Nrf2. Nrf2 binds to ARE in the nucleus and promotes the transactivation of the ARE coded genes (102). ITCs are known to be potent Nrf2 activators and exhibit anti-oxidative and anti-carcinogenic effects via the up-regulation of ARE driven genes (99). Even if the mechanism of interaction between ITCs and Keap1 is complicate and not fully understood, it might be mediated by activation of protein kinases, or as a direct reaction of ITCs with the sulfydryl groups of specific cysteine residues in Keap1, resulting in conformational changes that would drive the release of Nrf2 (103).

Similar to carcinogenesis, oxidative stress and chronic inflammation are central in the pathogenesis of PD and other neurodegenerative diseases, and the protective effects of isothiocyanates are evident in models of nervous tissue injury and neurodegeneration. Sulforaphane, one of the most studied ITCs, has protective effects on neurons of the central nervous system decreasing both microglial activation and the upregulation of inflammatory markers following endotoxin injection (104). However, there are few studies on the detailed mechanism of neuroprotective effects of isothiocyanates in the central nervous system (105). The neuroprotective effects of sulforaphane are accompanied by activation of the transcription factor Nrf2 and upregulation of its target genes. ITCs cross the cell membrane and accumulate in the cytoplasm where they bind to glutathione and other cellular thiols, reacting with the -SH groups. Although this reaction is reversible, it is believed to be the main driving force for accumulation of ITCs and for enzyme induction (106). Phase-2 enzymes are generally regarded as antioxidants as many of them have been proven to increase the cellular levels of antioxidant molecules like glutathione, or protect the cell from ROS and oxidizing species (107). For instance, sulforaphane was tested for its protective property in the ischemia reperfusion model, as representation for oxidative stress-mediated injury model, where the ITC dramatically induced phase 2 enzymes, decreasing the Keap1 protein levels and increasing Nrf2 nuclear translocation (106).

Wasabi (Wasabia japonica) is a member of the Brassicaceae family of vegetables, and its rhizome is a very popular pungent spice in Japan. Wasabi differs from other Brassicaceae species because it contains higher concentration of ITCs, especially long-chain ITCs. The bioactive components of wasabi have been identified as a series of ITC analogues, of which 6-(methylsulfinyl)hexyl isothiocyanate (6-MSITC) (Fig. 13) is a major active compound (102).

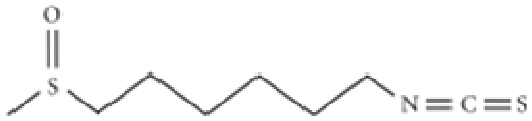


Figure 13. Chemical structure of 6-MSITC

In this contest appears the fundamental importance to investigate if 6-MSITC is able to interact and modify the progression of neurodegenerative disorders like PD.

2. AIM OF THE STUDY

PD is a pathology is characterized by a slow and progressive loss of dopaminergic neurons in SN, and represents the most frequent representation between movement diseases.

Also if the etiology of PD is still unclear, it is already strengthened the multifactorial origin of the pathology. Pathogenesis seems to be strongly related to oxidative stress, neurotoxicity of excitatory aminoacids and apoptotic mechanisms.

Pharmacological therapies are successful only for what that concern motorial symptoms, but they are not powerful to prevent or arrest disease's progression. In this context it is evident how important is the concept of neuroprotection, that aims to interfere with mechanisms subtending to cellular death. Iin the last decade, numerous works suggested the potential neuroprotective role of various phytochemical compounds such as cathechins of green tea, anthocyanes of red fruits and other polyphenols.

This work aims to study mechanisms of natural compounds to prevent or slow down neurodegenerative diseases. This study is focused on neuroprotective potential of 6-(methylsulfinyl)hexyl isothiocyanate (6-MSITC) in an experimental *in vivo* model of PD. The condition was induced by unilateral stereotaxic injection of 6-OHDA. For this purpose, the damage was provoked in STR, such as nigro-striatal terminations, that permitted to obtain a minor entity lesion and with a major time to establish. The lesion obtained spreads in a backward way and the slow evolution of the pathology permits to evaluate the potential efficacy of treatments to contrast damage induced.

This work has an integrated approach of behavioral assessment, biomolecular and immunohistochemistry techniques to evaluate neuroprotective effects of 6-MSITC.

In particular, we have used Rotarod test to assess motor coordination and rotational behavior induced by apomorphine injection, to estimate lesion's entity and the eventually recovery after the treatment.

After the sacrifice we used Western Blotting and immunohistochemistry to evaluate tyrosine hydroxylase levels in our samples. Furthermore, we evaluate the apoptotic process by analisys of DNA fragmentation and caspase-3 activation. After that we investigated how 6-MSITC is able to interact with cell's redox status and GSH system.

3. MATERIALS AND METHODS

3.1. ANIMALS AND EXPERIMENTAL DESIGN

Male C57Bl/6 (9 weeks old, 25–30 g body weight at the beginning of the experiment; Harlan, Milan, Italy) mice were housed 4-5 for cage under 12 h light/12 h dark cycle (lights on from 7:00 a.m. to 7:00 p.m.) with free access to food and water in a temperature (22±2°C) and humidity (60%) controlled room (Fig. 14). Experimental procedures were carried out in the light cycle (from 9:00 a.m. to 3:00 p.m.) including control groups in any tests utilized. In particular, the experimental protocol was based on the unilateral stereotaxic intrastriatal injection of 6-OHDA. Animals were randomly divided into 4 groups (n=10-12 per group), as follows: 6-OHDA/saline; 6-OHDA/6-MSITC; sham/saline; sham/6-MSITC. Two groups received a 6-OHDA injection in the left STR, while the other two received the same volume of saline solution (sham groups). Each mouse served as its own control, since left-sided levels (ipsilateral to the lesion) were always compared to right-sided levels (contralateral to the lesion) of the same animal. One hour after brain lesion, we started intraperitoneal (ip) administration of 5 mg/kg 6-MSITC (Lkt Laboratories, St. Paul, MN, USA) or vehicle (saline) in both lesioned and sham mice. We injected mice twice a week for four weeks. Four weeks after the lesion, we assessed the extent of the lesion using the rotational behavior test and we also evaluated motor function on the rotarod apparatus (Ugo Basile, Comerio, VA, Italy). At the end of behavioral analysis, mice were sacrificed by cervical dislocation to perform immunohistochemistry and neurochemical analysis.

All experiments were carried out in accordance with Directive 2010/63/EU and Directive 86/609/CEE and approved by the corresponding committee at the University of Bologna (PROT. n. 15-IX/9). Care was taken to minimize the number of experimental animals and to take measures to limit their suffering. Mice were allowed to acclimatize for at least 1 week before the start of experiments.

Figure 14. Mouse C57BL/6JOlaHad

3.1.1. NEUROTOXIC LESION

Animals were anesthetized under gaseous anesthesia (2% isoflurane in 1 L/min oxygen/nitrous oxide) using a gaseous anesthesia system (Ugo Basile, Varese, Italy) and then mice were positioned on a mouse stereotaxic frame (myNeuroLab, Leica-Microsystems Co, St. Louis, MO, USA). Anesthesia was maintained with 1.5% isofluorano/O_2 (1L/min). The scalp was incised to reveal the skull and recognize the bregma to set coordinates.

6-OHDA (Sigma-Aldrich, St. Louis, MO, USA; Fig. 15) was injected into the left STR, using the stereotaxic mouse frame and a 10 µL Hamilton syringe. 6-OHDA was dissolved at a concentration of 4 µg/µL saline in 0.02% ascorbic acid just before the use and 2 µL was injected at a rate of 0.5 µL/min.

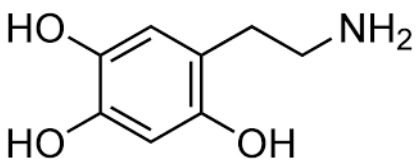


Figure 15. 6-OHDA structure.

The needle was left in place for 3 min after the injection before slow retraction, followed by cleaning and suturing of the wound (Histoacryl, Aesculap AG, Germany). Sham mice received the equivalent volume of saline into the left STR. The injection was performed at the following co-ordinates with a flat skull position:

AP + 0.50

ML - 2.00

DV - 2.50

After the surgery mice were collocated under an irradiating lamp to promote the recovery for the time required.

3.1.2. TREATMENT WITH 6-(METHYLSULFINYL)HEXYL ISOTHIOCYANATE

The treatment with 6-MSITC (6-(methylsulfinyl)hexyl isothiocyanate, LKT Laboratories, Inc., St. Paul, MN, USA) was carried out with ip administration of 5 mg/kg or vehicle in both lesioned and sham mice from 1 hour after the surgery and then two times a week for four weeks (Fig. 16).

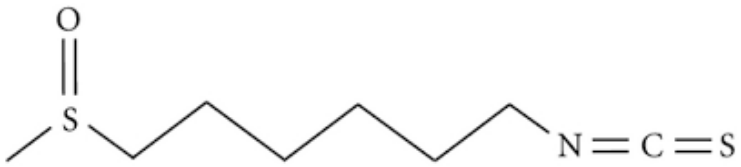


Figure 16. 6-MSITC structure

3.1.3. ROTAROD TEST

Rotarod test was used to evaluate the presence and severity of the lesion induced; the time that animals spent on the instrument is indicative of motor coordination and balance. The test was carried out using a commercially available mice rotarod apparatus (Ugo Basile, Varese, Italy; Fig. 17). The unit consists of a rotating spindle divided into 5 lanes by gray plastic dividers, a power source for turning the spindle and grids beneath the rotating roller where mice can safely fall, and the time latency to fall (s) is automatically recorded.

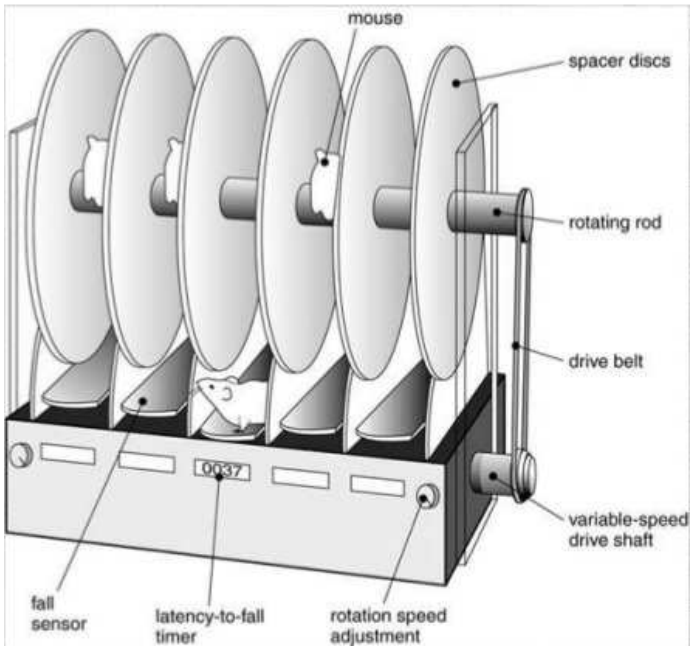


Figure 17. Rotarod

METHOD

Animals were transferred to the experimental room at least 1h before the test in order to let them acclimatize to the test environment. All scores were assigned by the same observer who was unaware of the animal treatment. The test was accomplished 4 weeks after the surgical procedure.

All mice were pre-trained for 2 days in order to reach a stable performance. The apparatus tests five mice at one time, with one mouse in

each section of the rod. Each animal was given three independent trials, each lasting 180 s (with a 20 min inter-trial period). Mice were mounted on the rod and the apparatus turned on to a fixed speed of 22 rpm. The latency of fall from the apparatus was recorded. Values were expressed as mean of retention time on the rotating bar over the three test trials.

3.1.4. ROTATIONAL BEHAVIOR

Apomorphine-induced rotations were determined 4 weeks after the surgical procedure (Sigma; Fig. 18). Apomorphine is a direct dopamine receptor agonist; it is able to induce contralateral rotation. This behavior suggests the presence of a different number of dopaminergic receptors in the nigro-striatal pathway in mice lesioned by 6-OHDA. Indeed, the dopaminergic agonist stimulates in a massive way the healthy hemisphere than the lesioned one. For this reason the roditor turns in the contralateral side, that is the healthy one.

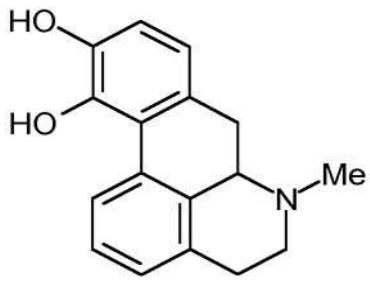


Figura 18. Apomorphine structure.

METHOD

Animals were transferred to the experimental room at least 1h before the test in order to let them acclimatize to the test environment. All scores were assigned by the same observer who was unaware of the animal treatment. The test was accomplished 4 weeks after the surgical procedure.

Briefly, mice received a subcutaneous injection of apomorphine (0.05 mg/kg saline). They were acclimatized in plexiglass cylinders for 5 min prior to testing. After apomorphine administration, full body ipsilateral and contralateral turns were recorded using an overhead videocamera over a

period of 10 minutes. Subsequently, each 360° rotation of the body axes was manually counted as a rotation. Values were expressed as mean of contralateral turns collected during 10 minutes.

3.1.5. SACRIFICE AND TISSUE PREPARATION FOR IMMUNOHISTOCHEMISTRY AND NEUROCHEMICAL ANALYSIS

Once behavioral analysis was completed, mice were deeply anesthetized and sacrificed by cervical dislocation and some of them were perfused with 4% of paraformaldehyde. The brains were removed and were immersed in the fixative solution for 48 h. The non-perfused brains were rapidly removed and placed into dry-ice. The right and left STR and SN were dissected on an ice-cold plastic dish. Samples were then snap frozen in liquid nitrogen, and kept at −80°C until analysis. Tissues were homogenized in lysis buffer (50 mM Tris, pH 7.5, 0.4% NP-40, 10% glycerol, 150 mM NaCl, 10 µg/ml aprotinin, 20 µg/ml leupeptin, 10 mM EDTA, 1 mM sodium orthovanadate, 100 mM sodium fluoride), and protein concentration was determined by the Bradford method.

3.2. WESTERN BLOTTING

Activation of tyrosine hydroxylase (TH) was evaluated by Western Blotting, useful technique to quantify proteins that recognize a specific antibody.

METHOD

Samples (30 µg proteins) were added to the *Loading Buffer 6x* (4x tris HCl/SDS, pH 6.8; glycerol; SDS; DTT; bromophenol blue) and then separated on 12% SDS-polyacrylamyde gels (Bio-Rad, Hercules, CA, USA) in *Running Buffer* (25 mM Tris; 192 mM Glycine; 0,1% (w/v) SDS pH 8,3) for 30 minutes at 200 V, and electroblotted onto 0.2 µm nitrocellulose membranes in *Blotting Buffer* (25 mM Tris; 192 mM Glycine; 20% (v/v) MeOH pH 8,3) for 1 hour at 100 V. After the transfer, membranes were incubated for 2 hours in *Block Solution* (5% no-fat powder milk; TBS; 0.05% Tween 20) to block aspecific binding sites. Membranes were incubated overnight at 4°C with primary antibody recognizing TH (1:1000; Millipore). The day after membranes were washed with TBS-T (TBS + 0.05% Tween20), and then incubated with a horseradish peroxidase (POD) linked anti-rabbit secondary antibody (1:2000; GE Healthcare, Piscataway, NJ, USA) for 1 hour at room temperature (RT). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA). The same membranes were stripped (100 mM β-mercaptoethanol; 2% SDS; 62,5 mM Tris pH 6.7) and reprobed with a β-actin antibody (1:1000; Sigma-Aldrich) 2 hours at RT and then incubated with a horseradish peroxidase (POD) linked anti-mouse secondary antibody (1:2000; GE Healthcare, Piscataway, NJ, USA) for 1 hour at RT. Data were analyzed by densitometry, using Quantity One software (Bio-Rad). Values were normalized to corresponding β-actin and expressed as fold increase versus respective contralateral intact site.

3.3. DNA FRAGMENTATION

The involvement of apoptotic process after the lesion induced was evaluated by the determination of cytoplasmatic histone-associated DNA fragments using the Cell Death Detection ELISAPLUS kit (Roche Diagnostics, Mannheim, Germany) and according to the protocol from the company. The assay is based on a quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes (histone-associated DNA fragments) in the fraction of tissue lysates (Fig. 19).

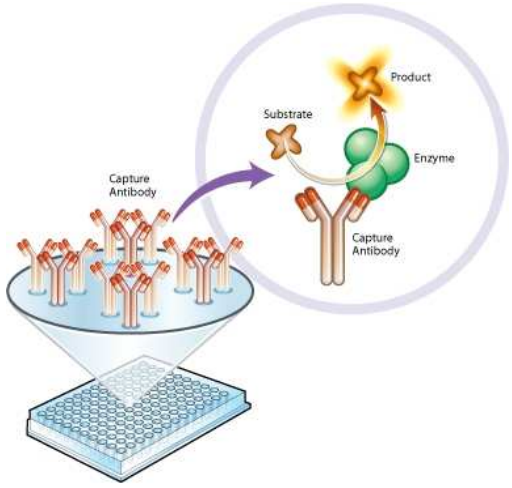


Figura 19. Mechanism of function ELISA kit.

METHOD

Aliquots with lysates corresponding to 80 µg of proteins were used at each reaction. The amount of nucleosomes demonstrating DNA degradation was quantified by POD retained in the immunocomplex. POD was determined photometrically at 405 nm with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as a substrate by a microplate reader (GENios, TECAN®, Männedorf, Switzerland) after 15 min of substrate reaction time. Values are expressed as mean of Optical Density (OD) of each experimental group.

3.4. CASPASE-3 ACTIVATION

Caspase-3 enzyme activity was determined using a protocol adapted by Movsesyan et al. (108). The assay is based on the hydrolysis of acetil-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) moiety by caspase-3 with the liberation of pNA (Fig. 20).

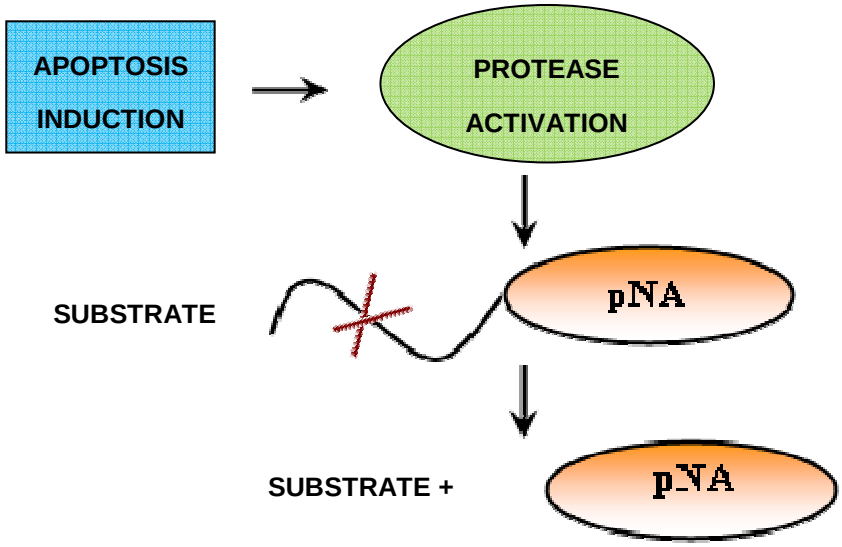


Figure 20. Caspase-3 activation analisys.

METHOD

Briefly, tissue lysates of SN were incubated with assay buffer (50 mmol/L Hepes, pH 7.4; 0.2% CHAPS; 20% sucrose; 2 mmol/L EDTA; and 10 mmol/L dithiothreitol) and a 50 µmol/L concentration of chromogenic pNA specific substrate (Z-Asp-Glu-Val-Asp-pNA; AlexisBiochemicals, San Diego, CA, USA). In a final volume of 100 µL (containing 120 µg of protein), each test sample was incubated for 3 h at 37°C. The amount of chromogenic pNA released was measured with a microplate reader (GENios, TECAN®) at 405 nm. Values are expressed as mean of Optical Density (OD) of each experimental group.

3.5. REDOX STATUS EVALUATION

The redox status, in terms of ROS formation, was measured as described previously (109), based on the oxidation of 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to 2′7′-dichlorofluorescein (DCF). Briefly, the reaction mixture (60 µL) containing 2 mg/mL of DCFH-DA was incubated for 30 min to allow the DCFH-DA to be incorporated into any membrane-bound vesicles and the diacetate group to be cleaved by esterases. After 30 min of incubation, the conversion of DCFH-DA to the fluorescent product DCF was measured using a microplate reader (GENios, TECAN®) with excitation at 485 nm and emission at 535 nm. Background fluorescence (conversion of DCFH-DA in the absence of homogenate) was corrected by the inclusion of parallel blanks. Values were normalized to protein content and expressed as mean of fluorescence intensity arbitrary units (UF) of each experimental group.

3.6. GLUTATHIONE CONTENT

GSH content was estimated using the protocol described earlier (110). Briefly, aliquots of 50 µL of samples were precipitated with 100 µL of sulfosalicylic acid (4%). The samples were kept at 4°C for at least 1h and then subjected to centrifugation at 3000 rpm for 10 min at 4°C. A volume of 25 µl of the assay mixture and 50 µL of 5-5'-dithio-bis (2-nitrobenzoic acid) (4 mg/mL in phosphate buffer, 0.1 M, pH 7.4) was made up to a total volume of 500 µL. The yellow color that developed was read immediately at 412 nm (GENios, TECAN®) and results were calculated using a standard calibration curve. Values are expressed as mmol GSH/mg of total lysate proteins per assay.

3.6.1. GLUTATHIONE-S-TRANSFERASE AND GLUTATHIONE REDUCTASE ACTIVITIES

GST activity was assessed by the ability to conjugate GSH to 1-chloro-2,4-dinitrobenzene (CDNB), as previously shown (111). Tissue lysates were added to 0.1 mol/L potassium phosphate 1 mmol/L EDTA with 20 mmol/L GSH and 20 mmol/L CDNB. The rate of appearance of the GSH-CDNB conjugate was measured at 340 nm (GENios, TECAN®).

GR activity was determined using the Glutathione Reductase Assay Kit according to the manufacturer's instructions (Sigma-Aldrich). Briefly, the assay mixture consisted of phosphate buffer (pH 7.6), EDTA (1 mM), oxidized GSH (2 mmol/L) and NADPH (2 mmol/L). GR was determined by measuring the disappearance of NADPH at 340 nm (GENios, TECAN®).

The specific activities of GST and GR are expressed as enzyme units mL-1/mg of total lysate proteins per assay.

3.7. IMMUNOISTOCHEMYSTRY

Fixed brains were sliced on a vibratome (Leika Microsystems, Milan, Italy) at 40 µm thickness. After deparaffinization, endogenous peroxidase was quenched with 3% hydrogen peroxide (H_2O_2). Non-specific adsorption was minimized by incubating the section in 10% normal goat serum for 30 min. Sections were then incubated overnight at 4°C, with a rabbit anti-TH antibody (1:500; Millipore, Temecula, CA, USA), rinsed in TBS, and re-incubated for 1 h, at RM, with a goat biotinylated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA). Finally, sections were processed with the avidin-biotin technique and reaction products were developed using commercial kits (Vector Laboratories). To verify the binding specificity, some sections were also incubated with only primary antibody (no secondary) or with the secondary antibody (no primary). In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all experiments carried out.

Image analysis was performed by a blinded investigator, using an Axio Imager M1 microscope (Carl Zeiss, Oberkochen, Germany) and a computerized image analysis system (AxioCam MRc5, Zeiss) equipped with dedicated software (AxioVision Rel 4.8, Zeiss). After defining the boundary of the SN at low magnification (2.5x objective), the number of TH-positive cells in the SN was counted bilaterally on at least four adjacent sections at a higher magnification (40x objective). Neuronal survival in the SN was expressed as the percentage of TH-positive neurons on the lesioned side, with respect to the contralateral, intact side. In the absence of a stereological count, this approach was chosen to avoid methodological biases due to interindividual differences, and has been previously used to assess the extent of 6-OHDA-induced lesion in the SN (112). The intensity of TH immunoreactivity in STR was measured using AxioVision Rel software (Zeiss) and results were expressed as percentage of TH density on the lesioned side with respect to the contralateral side.

3.8. STATISTICAL ANALYSIS

Data are reported as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc test. Differences were considered significant at $p<0.05$. Analyses were performed using PRISM 5 software (GraphPad Software, La Jolla, CA, USA).

4. RESULTS

Neuroprotective effects of 6-MSITC were evaluated in a mouse model of PD by intracranical injection of 2 μL of 6-OHDA at 4 μg/μL in the left STR of C57BL/6 mice. 6-MSITC (5 mg/kg) was injected by ip, from one hour after the surgery twice a week until the end of the experiment (4 weeks). Animals were divided in four experimental groups: one group has received physiological solution and was treated with the same solution (Sham/saline); one group has received physiological solution and then was treated with injection ip of 6-MSITC (Sham/6-MSITC); one group was lesioned with 6-OHDA and then has received physiological solution as treatment (6-OHDA/saline); and finally one group that has received intracranical injection of neurotoxin and then was treated with 6-MSITC (6-OHDA/6-MSITC).

Initially we conducted a behavioral investigation after 6-MSITC treatment in our model. In order to do this, we used Rotarod to test animal's ability to coordinate their movements on the rolling rod. The time taken to stay on the rotating rod was significantly decreased ($p<0.05$) in the 6-OHDA/saline group when compared with the Sham/saline group, while no significant difference in motor coordination was observed in the Sham/saline group as compared to the 6-OHDA/6-MSITC group. Moreover, the latency time to fall off was signifintally increased in 6-OHDA/6-MSITC group with respect to 6-OHDA/saline group ($p<0.05$; Fig. 21a). Behavioral quantification of dopamine depletion, done by apomorphine-induced rotations 4 weeks after the lesion, demonstrated a significant increase in the number of apomorphine induced rotations in lesioned mice compared with sham groups ($p<0.001$ Sham/saline vs 6-OHDA/saline; $p<0.05$ Sham/saline vs 6-OHDA/6-MSITC). More interestingly, statistical analysis of the total rotations, showed that the 6-OHDA/6-MSITC group exhibited a significant decrease of asymmetric motor behavior compared to the 6-OHDA/saline group ($p<0.05$; Fig. 21b)

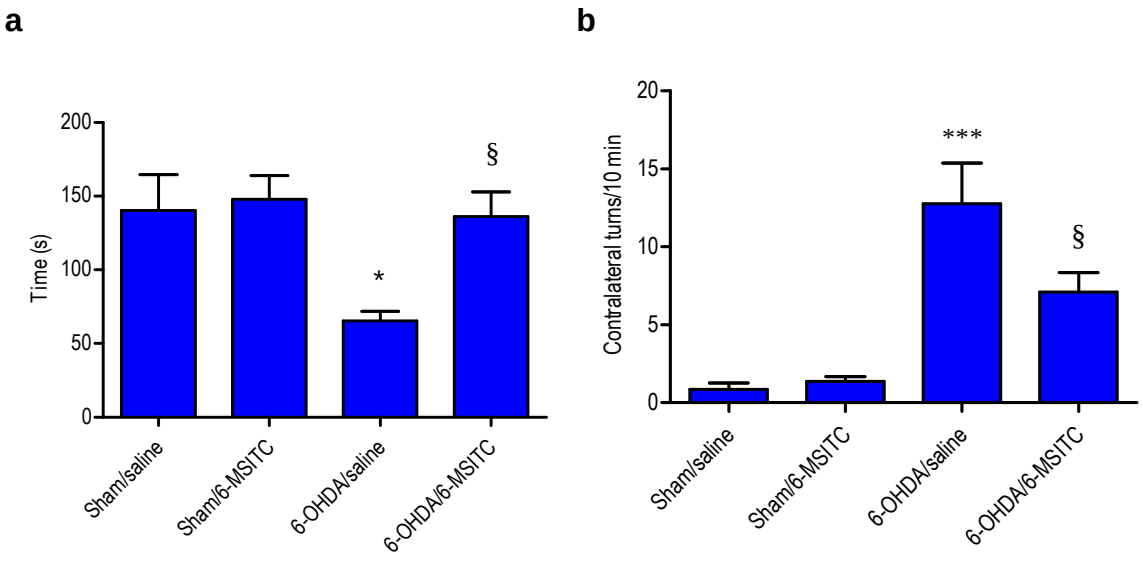


Figure 21. Effects of 6-MSITC on motor coordination and apomorphine-induced rotational behavior in 6-OHDA mice. a. The latency to fall (s) from rotarod spindle at constant speed (22 rpm-cut off 180 s) was recorded. Values are experessed as mean±SEM (n=10) of retention time (s) on the rotating bar. **b.** the number of ipsi and contralateral rotations was counted for 10 min. values are expressed as mean±SEM (n=10) of contralateral turns collected during 10 minutes. (**a:** *p<0.05, 6-OHDA/saline vs sham groups; §p<0.05, 6-OHDA/6-MSITC vs 6-OHDA/saline; **b:** ***p<0.001, 6-OHDA/saline vs sham groups; §p<0.05, 6-OHDA/6-MSITC vs 6-OHDA/saline and saline groups; ANOVA, post-hoc test Bonferroni).

Four weeks after the lesion, mice were sacrificed; SN and STR tissues were extracted and we proceeded with biomolecular analysis. In first instance, we evaluated tyrosine hydroxylase (TH) levels in SN e STR, to evaluate dopamine synthesis. By immunohistochemical analysis we demonstrated that lesioned animals showed a consistent reduction of TH-positive cells in the left SN, compared to the intact side, with a consistent neuronal loss (p<0.001; Fig. 22a). 6-MSITC treatment helped to preserve the integrity of nigrostriatal tract producing a significant decrease of dopaminergic cell loss in the SN (p<0.01 6-OHDA/saline vs 6-OHDA/6-MSITC).

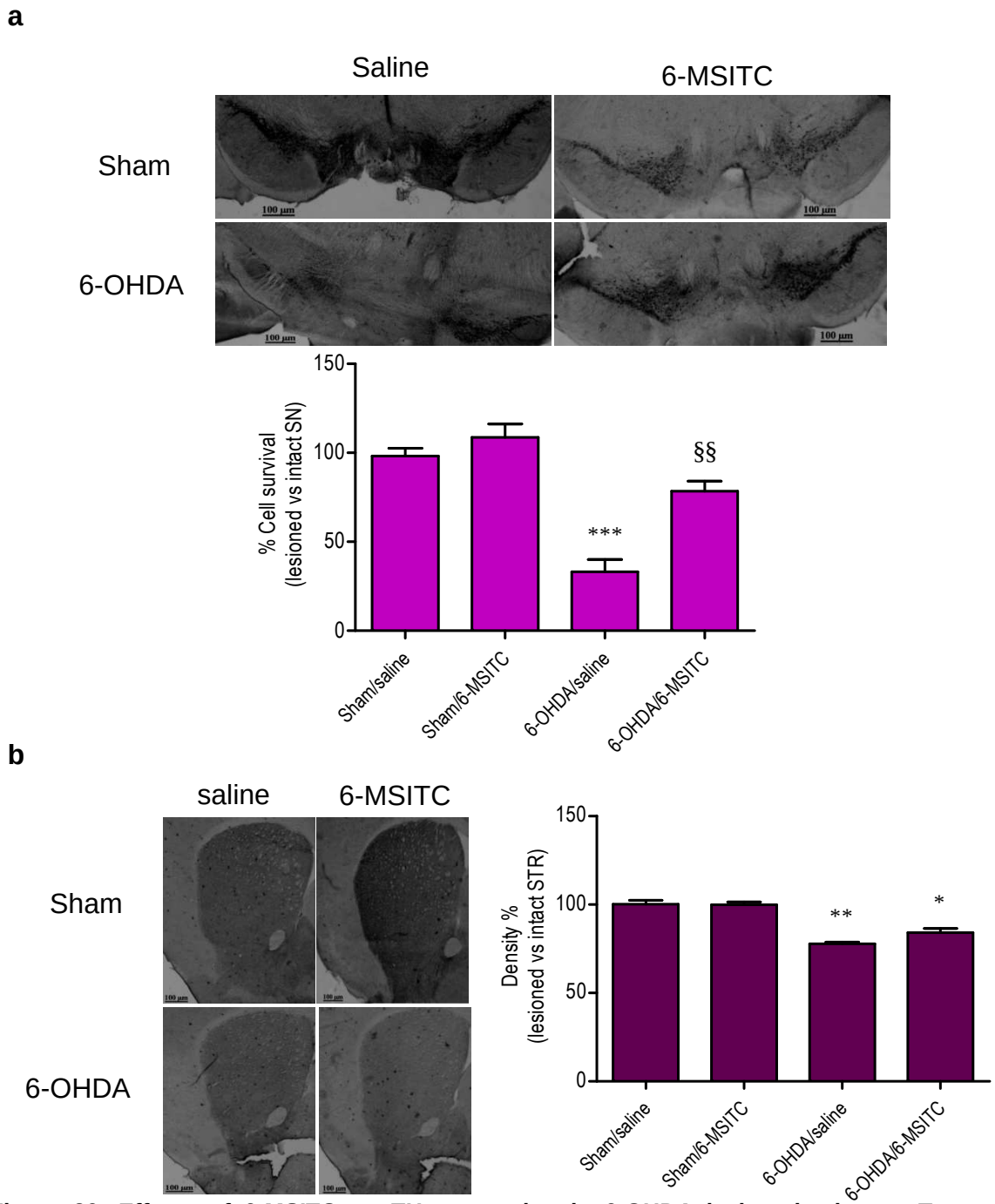


Figure 20. Effects of 6-MSITC on TH expression in 6-OHDA lesioned mice. a. Top: representative photomicrographs of brain coronal sections containing both ipsilateral and contralateral SN in different treatments groups. Scale bar 100 µm. **Bottom:** histogram representing dopaminergic cell survival in the SN. **b. Left:** representative photomicrographs of STR sections in different treatments groups. Scale bar 100 µm. **Right:** quantification of striatal TH fiber densities. Values are expressed as mean±SEM (n=10) of the percentage of surviving TH-positive cells (a) or density (b) of the lesioned side compared to the intact hemisphere (**a:** ***p<0.001, 6-OHDA/saline vs sahm groups; §§p<0.01, 6-OHDA/6-MSITC vs 6-OHDA/saline; **b:** **p<0.01, 6-OHDA/saline vs sham groups; *p<0.05, 6-OHDA/6-MSITC vs sham groups; ANOVA, post-hoc test Bonferroni).

As shown in figure 22b, immunohistochemical detection of TH fiber staining in striatal tissue sections revealed that 6-OHDA reduced significantly the TH immunostaining in dopaminergic terminals ($p<0.01$). Although 6-MSITC slightly ameliorated the loss of TH fibrous staining, the 6-OHDA/6-MSITC group was significantly different from sham groups ($p<0.05$).

We also performed Western Blotting analysis for TH protein that confirmed what we have seen before with the immunostaining. 6-OHDA treatment induced a significant loss of TH immunoreactivity in the SN and STR ($p<0.05$; Fig. 23a and 23b), which was completely attenuated in the SN by 6-MSITC treatment ($p<0.05$; Fig. 23a).

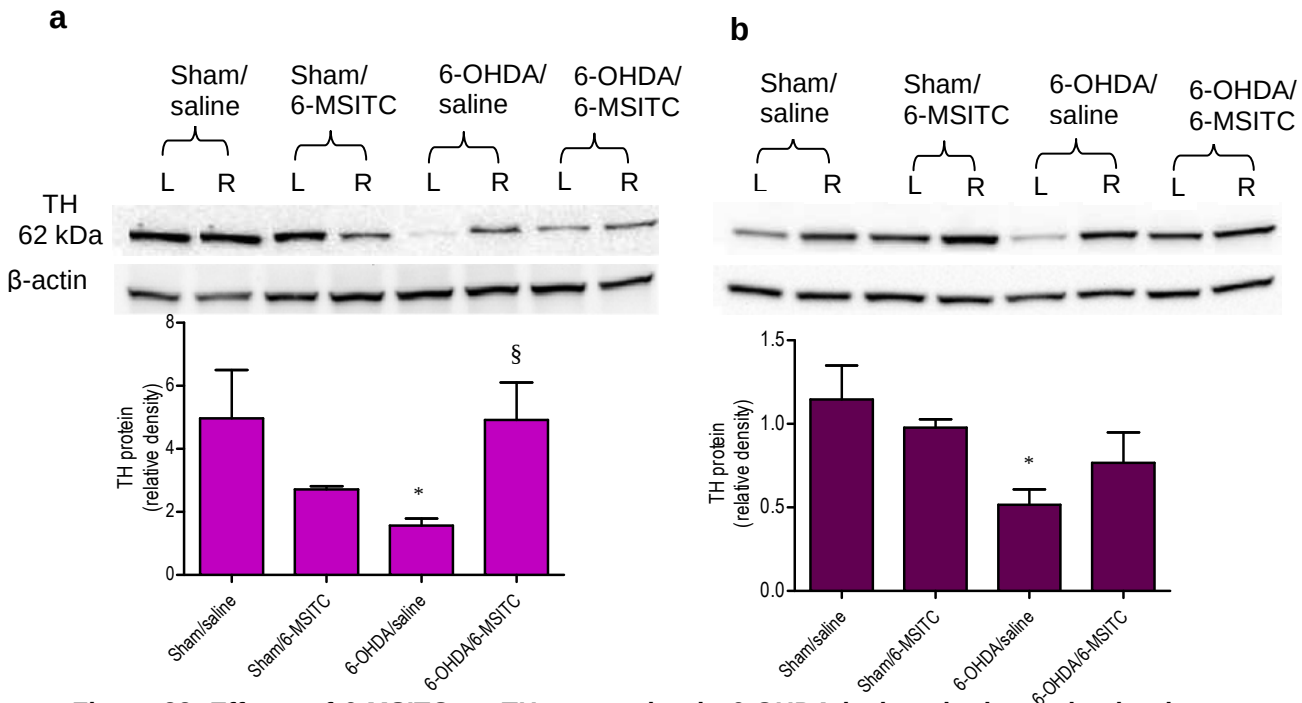


Figure 23. Effects of 6-MSITC on TH expression in 6-OHDA lesioned mice, valuation by Western Blotting. a, b. Top: Representative images of the protein expression in SN (a) and in STR (b). **Bottom:** Quantitative analysis of the Western Blot results for TH levels in SN (a) and STR (b). The graphs show densitometry analysis of the bands appertaining to the protein of interest. The values result from normalization of the ratio between the density of the band of interest and the density of β-actin, compared to the corresponding ratio in the intact side. Values are expressed as mean of fold increase±SEM (n=10) (**a:** *$p<0.05$, 6-OHDA/saline vs Sham/saline; $^{§}p<0.05$, 6-OHDA/6-MSITC vs 6-OHDA/saline; **b:** *$p<0.05$ 6-OHDA/saline vs Sham/saline; ANOVA, post-hoc test Bonferroni).

Subsequently, we investigated cell death caused by 6-OHDA, through the analysis of DNA fragmentation and caspase-3 activation. To quantify DNA fragmentation, we used an ELISA assay which measures low molecular weight histone-associated DNA. In our experimental model, 6-OHDA injection caused an increase of DNA fragmentation in SN samples compared to sham groups ($p<0.01$, Fig. 24a). More interestingly, 6-MSITC significantly blocked 6-OHDA-induced DNA fragmentation ($p<0.01$). We confirmed this result through the evaluation of caspase-3 activation by a colorimetric assay. As shown in figure 24b, following the injection of 6-OHDA, we detected a robust activation of the main effector of apoptotic cell death in SN sample ($p<0.01$). Concordantly with its inhibition of DNA fragmentation, 6-MSITC significantly blocked the 6-OHDA-induced caspase-3 activation ($p<0.05$).

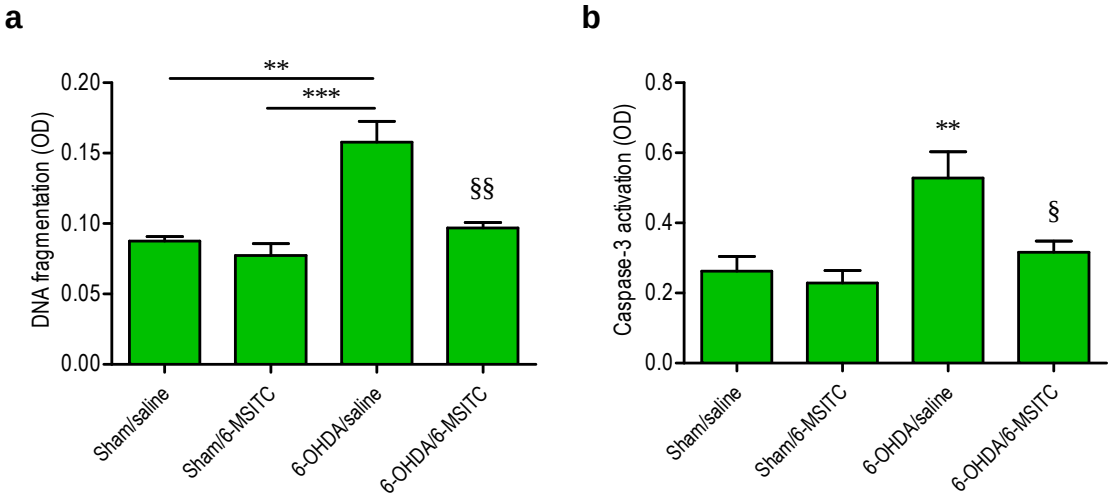


Figure 24. Effects of 6-MSITC on DNA fragmentation and caspase-3 activation in SN of 6-OHDA lesioned mice. a. 6-OHDA injection significantly increased DNA fragmentation in lesioned mice compared to sham groups, on the other hand, 6-MSITC treatment is able to reduce the levels of DNA fragmentation. **b.** 6-OHDA injection significantly increased caspase-3 activation, while 6-MSITC treatment is able to counteract this effects. Values are expressed as mean±SEM (n=10) of optical density (OD) of each experimental group (**a:** ***$p<0.001$, 6-OHDA/saline vs Sham/6-MSITC; **$p<0.01$, 6-OHDA/saline vs Sham/saline; $^{§§}p<0.01$, 6-OHDA/6-MSITC vs 6-OHDA/saline; **b:** **$p<0.01$, 6-OHDA/saline vs sham groups; $^{§}p<0.05$, 6-OHDA/6-MSITC vs 6-OHDA/saline; ANOVA, post-hoc test Bonferroni).

We also investigated the potential effects of 6-MSITC on the cellular redox status, because ROS generated in PD brains trigger oxidative cellular

stress and consequent cell death in dopaminergic neurons. As an index of redox status, we utilized the fluorescent probe 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) to measure ROS levels in the SN. As shown in figure 25a, neurotoxin intrastriatal injection induced a significant change in redox status ($p<0.001$), while this effect was efficiently contrasted by 6-MSITC administration ($p<0.001$).

Furthermore, we decided to investigate endogenous antioxidant balance by the activation of GSH system. GSH system is responsible for neutralization of peroxide and maintenance of protein thiols in the reduced state. GSH content resulted significantly decreased in the 6-OHDA/saline group as compared to the Sham/saline group ($p<0.05$; Fig. 25b); in turn 6-MSITC consistently protected against the oxidative stress induced by 6-OHDA, in this way is able to maintain GSH close to baseline values ($p<0.05$).

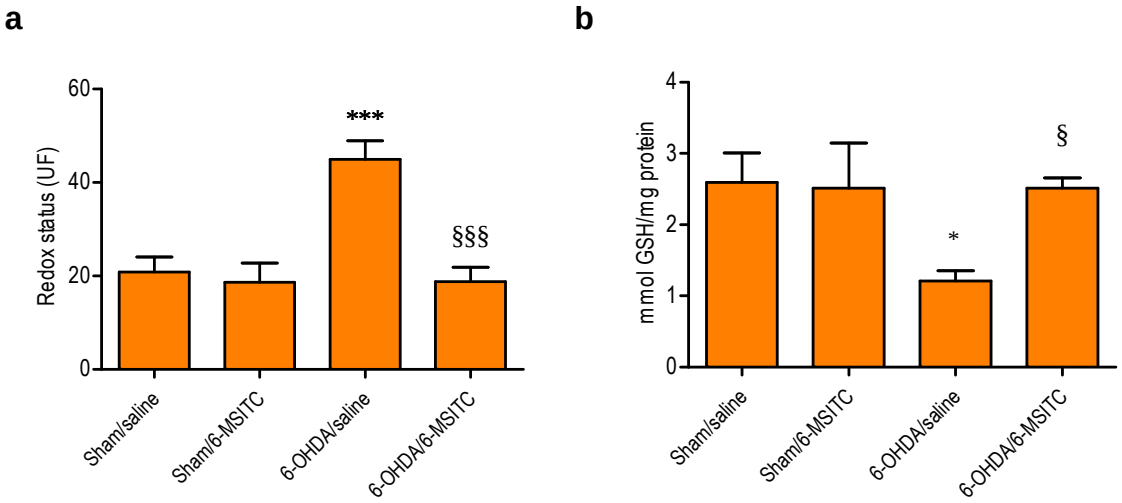


Figure 25. Effects of 6-MSITC on redox status and GSH content in SN of 6-OHDA lesioned mice. a. 6-OHDA injection induced a consistent increase in oxidative stress that it is reestablished by 6-MSITC treatment. Values are expressed as mean±SEM (n=10) of fluorescence intensity arbitrary units (UF). **b.** GSH content resulted significantly decreased after the lesion and the treatment with 6-MSITC is able to restore baseline levels. Values are calculated using a standard calibration curve and expressed as mmol GSH/mg protein. (**a:** ***$p<0.001$, 6-OHDA/saline vs sham groups; $^{§§§}p<0.001$, 6-OHDA/6-MSITC vs 6-OHDA/saline; **b:** *$p<0.05$, 6-OHDA/saline vs sham groups; $^{§}p<0.05$, 6-OHDA/6-MSITC vs 6-OHDA/saline; ANOVA, post-hoc test Bonferroni).

Finally, in view of the importance of GSH-dependent antioxidant enzymes, such as glutathione-S-transferase (GST) and glutathione reductase (GR), for cellular defense against oxidative damage, we investigated the activities of these enzymes. Especially, the primary role of GST is to detoxify xenobiotics by catalyzing the nucleophilic attack by GSH, in order to inactivate a number of oxidising species. The enzyme GR is also important in GSH homeostasis: it regenerates GSH from the oxidized form (GSSG). We observed that 6-OHDA/saline group showed alterations of brain antioxidant status compared to sham group with a decrease of GST activity ($p<0.01$; Fig. 26a) and an increase in 6-OHDA/6-MSITC group ($p<0.05$). The evaluation of GR activity demonstrated the same results, with a decreasing activity in 6-OHDA/saline group compared to sham group ($p<0.01$; Fig. 26b), and the consequent increase after the 6-MSITC treatment ($p<0.05$).

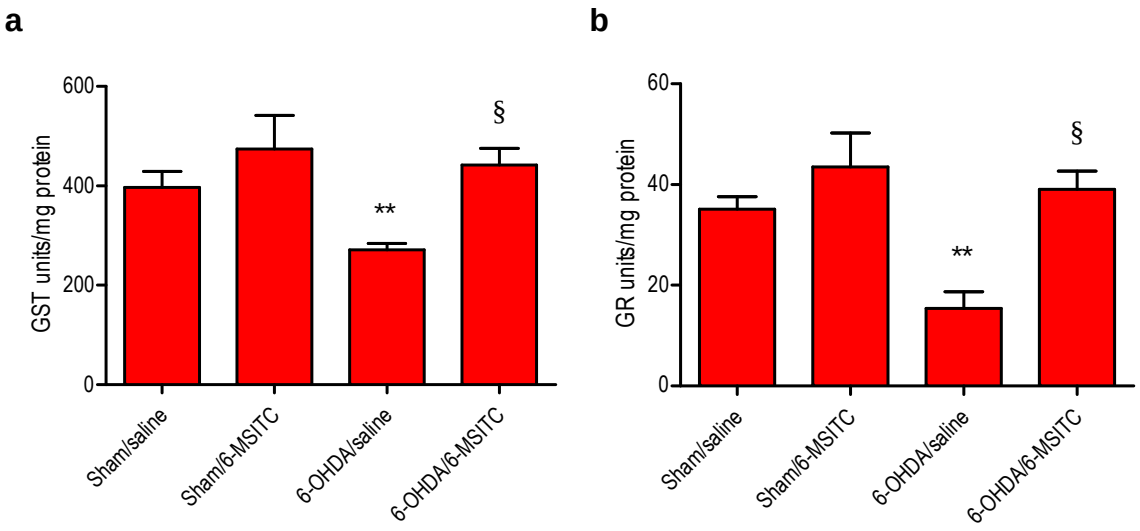


Figure 26. Effects of 6-MSITC on GST and GR activities in SN of 6-OHDA lesioned mice. The 6-OHDA intrastriatal injection determined a significant decreased of GST and GR activities that were reestablished by 6-MSITC treatment. Values are expressed as mean±SEM (n=10) of enzyme units/mg of proteins. (**a:** **$p<0.01$, 6-OHDA/saline vs Sham/6-MSITC; $^{§}p<0.05$, 6-OHDA/6-MSITC vs 6-OHDA/saline; **b:** **$p<0.01$, 6-OHDA/saline vs sham groups; $^{§}p<0.05$, 6-OHDA/6-MSITC vs 6-OHDA/saline; ANOVA, post-hoc test Bonferroni).

5. DISCUSSION

One of the major bioactive component in wasabi roots is the isothiocyanate 6-MSITC (105). Several studies have shown that 6-MSITC is rapidly accumulated to very high levels in a variety of cell types, through its conjugation with cellular GSH (113). Animal experiments have indicated that 6-MSITC, such as its analogues, can be absorbed, reach micromolar concentration in the blood and accumulate in tissues where it is maintained to achieve protective effects (114). In addition, many in vivo studies suggest the ability of sulforaphane, a 6-MSITC analogue, to reach CNS were it could display protective effects. In this context, the isothiocyante sulforaphane presents many advantages, such as the potential ability to penetrate the BBB and deliver its neuroprotective effects in the CNS with a good pharmacokinetics (1). On the basis of its structural similarity to sulforaphane, we decided to study the neuroprotective effects of 6-MSITC.

The pathogenesis of many neurodegenerative diseases, including PD, may involve the generation of ROS, which causes oxidative stress (36). Oxidative stress and the depletion of GSH are both early biochemical events associated with PD. In particular, the loss of GSH and the impairment of the antioxidant systems based on GSH have been shown to trigger active dopaminergic cell death (41).

This study demonstrated that the preventive effect of 6-MSITC is closely related to the protection of nigrostriatal dopaminergic neurons from the neurotoxicity induced by 6-OHDA in the mouse brain. Furthermore, the present results suggest that the activation of the GSH-dependent antioxidant systems is involved in the neuroprotective effects of 6-MSITC.

In views of our findings, the levels of GSH, GR and GST in the SN of 6-OHDA-lesioned mice are obviously lowered, and this progressive reduction in antioxidant scavenging capacity induced by 6-OHDA is well documented

in the literature (115). 6-MSITC treatment strongly decreased ROS formation and increases GSH levels and GR and GST activities in the SN when compared with the 6-OHDA/saline group, indicating that the inhibition of the oxidative stress response in 6-OHDA-lesioned mice is one of the main mechanisms involved in its neuroprotective effect. These results are supported by Mizuno et al (105), who have shown that 6-MSITC prevents the induction of oxidative stress by cytotoxicity in rat striatal cultures by raising the intracellular GSH content via an increase in γ-glutamylcysteine synthetase expression, induced by the activation of the Nrf2-ARE detoxification pathway.

Alteration in the GSH/GSSG ratio can negatively impact the structural and mechanistic integrity of the cells, thereby impairing brain function and, ultimately, neuronal viability. GSH not only remove ROS but also directly or indirectly regulates the activity of a number of key enzymes (GSTs, glutathione peroxidases, and lipoxygenases), that modulate cell survival (116).

The concept that the 6-OHDA intrastriatal injection causes dopaminergic neurons death and dysfunction in the SN is mainly supported by the decrease in the number of TH immunoreactive cells was sustained by Blandini et al. who asserted that TH decrease might reflect a loss of phenotype, so dopaminergic cells are present but no longer able to express TH, rather than the real damage to cells and terminals (112). For this reason, we evaluated the induction of apoptosis in our experimental model and we found that 6-OHDA induced toxicity that involves the activation of caspase-3, which in turn activates the enzyme responsible for apoptotic DNA fragmentation. Based on these results, we can suggest that the decrease of caspase-3 activation and DNA fragmentation are strongly involved in the 6-MSITC mechanism of neuroprotection.

The preservation of functional nigral neurons contributed to the reduction of behavioral abnormalities induced by 6-OHDA. Our results demonstrate that hemiparkinsonian mice exhibited significant changes in

apomorphine-induced rotation after 6-OHDA intrastriatal administration. 6-MSITC induced a partial recovery in the rotational behavior test, in fact we still found a significant difference between the 6-OHDA/6-MSITC group and the sham operated mice. However, the 6-OHDA/6-MSITC group displayed a significant reduction in apomorphine-induced rotations as compared to the 6-OHDA/saline group. The rotarod test has been used as a drug free test for unilaterally 6-OHDA-lesioned animals to assess coordination and balance that permits to use for detection in loss of TH-immunoreactive cells in the SN (117). Our results showed that the time spent on the rotating rod was lower in 6-OHDA-lesioned mice, and more interesting, that 6-MSITC treatment significantly improved mice's performance.

It is important to underlie that 6-MSITC induced in TH immunostaining an increased expression in the SN but had no effect in the STR. Probably the absence of protection in the STR reflects the lack of complete recovery shown in rotational behavior test. Indeed, it is known that nigral dopaminergic neurons release dopamine not only from their axons projecting to the STR but also from their dendrites (118). Based on these considerations, 6-MSITC could strengthen the dopamine release demonstrating a crucial role in dopaminergic activity in the SN.

Taken together, the results of the present study highlight that the administration of 6-MSITC for one month is able to exert neuroprotective effects in the 6-OHDA model of PD. 6-MSITC treatment results in a significant decrease in oxidative stress and apoptotic cell death, leading to an improvement of behavioral impairments, in particular motor deficits.

It is known that oxidative stress is involved in both the initiation and the progression of PD (36, 40). At this regard, although the greater part of dopaminergic neuron loss has already occurred at the time of diagnosis of PD, maintenance of the redox status of the remaining dopaminergic neurons could slow the progression of this neurodegenerative disease. In conclusion, our results suggest that this isothiocyanate may be a promising neuroprotective compound against neurodegeneration that occurs in PD.

PART II

1. INTRODUCTION

1.1. CEREBRAL ISCHEMIA

More than any other organ of the body, brain integrity depends on the continuous blood supply of oxygen and glucose for covering the energy demands of the tissue. Cessation or severe reduction of blood flow results in a rapid biochemical and functional deficits which become irreversible unless blood flow is promptly restored.

With regard to the pathophysiology of cerebral ischemia, it can distinguish three major categories of flow reduction: transient global ischemia, permanent or transient focal ischemia, and microembolism. The most important clinical cause of global ischemia is cardiac arrest which induces a complete cessation of cerebral blood flow. Global ischemia also results from strangulation, severe shock or intracranical hypertension, but under these conditions flow decline is incomplete and heterogeneous. Focal brain ischemia is most frequently caused by thrombotic occlusion of the middle cerebral artery. Finally, microembolism, leading to multiple ischemic microfoci is caused by fat microemboli after bone fractures, by release of platelet aggregates and thrombotic materials from ulcerating atherosclerotic plaques or by air bubbles during cardiac surgery.

All these ischemic conditions exhibit different pathophysiology and require different therapeutic approaches (119).

In focal ischemia, the reduction of blood flow is most severe in the center of the region of the occluded artery. This is basically different from the incomplete global ischemia in which ischemia is most pronounced in the peripheral borderzone (120). The size of the ischemic region depends on the

efficacy of collateral blood supply. In the brain there are three major vessels – anterior, middle and posterior cerebral arteries – interconnected by the pial network of Heubner's anastomoses. Blood supply by this system depends on the anatomical configuration of the network, the vascular tone, blood viscosity and blood pressure (121). Under adverse conditions, ischemia may develop in the total distribution of the occluded vessel (maximal infarct), but the ischemic region may also be very small when collateral blood supply is optimal (minimal infarct) (122).

After an ischemic episode, is frequent that some brain regions are going to lose their property and the ability to work properly. In this contest appears clearly the importance of a rapid and efficient way of intervention, but also the importance of an efficient neuroprotective system able to prevent or at least to limit the damage bounded to a cerebral stroke event.

As enunciated before, there are two main types of stroke: hemorrhagic and ischemic (Fig. 27).

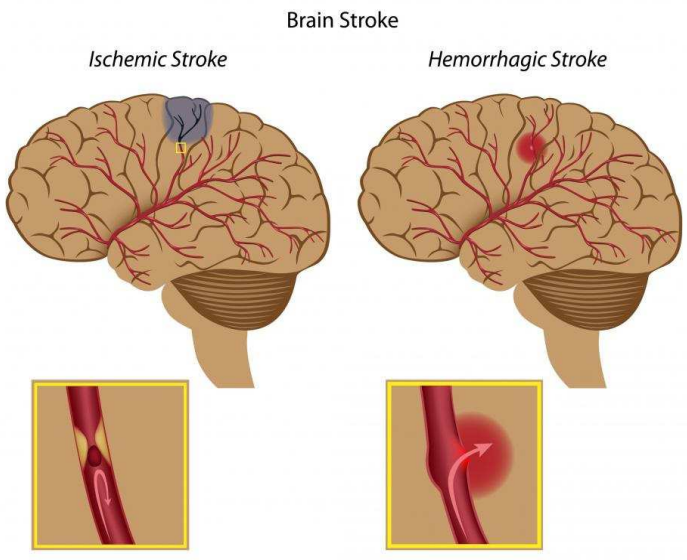


Figure 27. Ischemic and hemorrhagic strokes. On the left is showed a representation of ischemic stroke, after blood vessel blockage, there is a lack of blood flow to affected the area. On the right is represented an example of hemorrhagic stroke where the vessel's rupture is responsible of a leakage of blood.

The first case can results from a variety of conditions including uncontrolled hypertension and aneurysm. The hemorrhagic strokes in turn could be intracerebral or subarachnoid, which are associated with brain tissue damage by the rupture of a cerebral artery within or on the surface of the brain, respectively. On the other hand there are two kind of ischemic stroke: thrombotic and embolic; together the two types represent approximately 85% of all cases of strokes. Usually thrombotic strokes occur in the night or early in the morning because of a thrombus forming in an artery inside or leading to the brain mainly as a result of atherosclerosis. This type of stroke is further classified in lacunar and non-lacunar strokes. Lacunar infarcts are small and present a diameter minor of 20 millimeter usually are observed in the deep cerebral white manner or basal ganglia probably for the occlusion of a single small artery feeding the subcortical areas of the brain. For what that concern the embolic strokes, they occur when an embolus travels through the bloodstream until it enters in a vessel that is too tight to let it pass. The consequence is the complete absence of blood supply in the region of the brain usually feed by the vessel damaged (123).

1.1.1.INCIDENCE AND EPIDEMIOLOGY

Ischemic stroke is the third leading cause of death and the main cause of permanent adult disablement in industrialized countries (124). Also called brain attack, victims may suddenly manifest paralysis, impaired speech, or loss of vision due to the interruption of blood flow caused by thrombosis or embolism (125). Less frequently (less than 15% of cases), strokes are caused by hemorrhage or cardiac arrest. In the USA, as in other industrialized countries, stroke rates, adjusted for age, declined over the last 30 years (126). On average, in the USA strokes hit every 40 seconds and cause death every 4 minutes with an estimated death rate in 2007 of 41,6%. With the aging population these numbers may stabilize or increase over the next two decades. Among survivors, work capacity is compromised in 70% of patients, and 30% need assistance with self-care. From this point of view, the cost for stroke is a really massive problem. Changes in population demographics and overall risk of disease will place demands on health services for both acute stroke care and long-term care associated with more severe loss of function. New risk scores can risk-stratify patients presenting stroke and provide insights into the likely hood of long-term disability (127). In 2010 the estimated rate was 73.7 billion dollars for USA and projected to be 1.52 trillion dollars in 2050. This situation is a global mankind problem, and no racial or ethnic groups are spared. Developing industrialization of Asia and Africa is increasing unhealthy lifestyles, which promote stroke and other cardiovascular disease. As a result, the highest rates of stroke mortality and long-term disability occur in Asia, Russia, and Eastern Europe (128). Stroke is increasing rapidly in Eastern Europe and Central Asia compared with Western Europe and the United States (126). In China, rates of stroke and other cardiovascular disease are projected to increase dramatically due to combination of an aging population and the high prevalence of smoking and hypertension, for these reasons the estimated death rates are five to ten times higher than in USA (129). The types of stroke are also changing in rapidly developing Asian countries such as

China, with an increase in ischemic stroke and a decline in hemorrhagic stroke likely what seen in industrialized countries (130). Risk factor management is extremely important for the primary and secondary prevention of stroke in Asia as it is Western countries. In a meta-analysis of several Japanese studies of patients with non-cardiac sources of stroke, systolic and mean blood pressure were related to the risk of hemorrhagic and ischemic stroke and represent first targets for prevention programs (131). Ischemic stroke is a manifestation of atherosclerosis, a disease that affects all major arteries in the body. It is not surprising that recent studies in industrialized communities show fairly high rates of asymptomatic coronary disease in patients with stroke (132).

For that matter gender difference in the pathology, recent research demonstrates that women are disproportionately affected by stroke. Indeed it is the third leading cause of death for women, compared to the fifth leading cause of death for men (133). In the United States, there are currently 26% more female stroke survivors than male and this disparity is expected to increase as the aging population continues to expand, by the fact that the life expectancy is longer for woman than for men (134). Women have a lower incidence of ischemic stroke than men across most age groups, but in the highest age group (85 years of age) women have higher stroke incidence (135). While social factors certainly play a significant role, men and women exhibit a wide variety of biological variances that may contribute to this disparity, including differences in genetics, hormonal factors and immune response.

1.1.2.ORIGIN AND RISK FACTORS

A stroke risk factor is a characteristic of an individual that increases the risk for stroke compared to someone without that characteristic (136). Several risk factors for stroke have been identified by epidemiological studies. Some of these can be modified through pharmacological or non-pharmacological interventions and their identification is crucial to primary and secondary stroke prevention. Well documented modifiable risk factors for stroke are related to cardiovascular system such as arterial hypertension, some heart diseases, left ventricular hypertrophy, carotid stenosis, transient ischemic attack. Also metabolic conditions can play an important role like diabetes mellitus, hyperhomocysteinemia; not less important is lifestyle, and in this way is evident how cigarette smoking, alcohol abuse and limited physical activity have a fundamental role in stroke incidence. Other probable risk factors still not well documented so fare include some other heart diseases, plaques of the aortic arch; hormonal variations, such as use of oral contraceptives or hormonal replacement therapy. By the way also migraine, anti-phospholipid antibodies and hemostasis factors showed to be probable risk factors. For what that concern lifestyle, infections, drug use and air pollution, they have shown a correlation still not confirmed. Finally lipid metabolism plays a crucial role with dyslipidemia, metabolic syndrome and obesity. Hypercholesterolemia, the best documented modifiable risk factor for coronary artery disease, is incompletely defined. These risk factors often coexist, and they have been estimated to account for 60-80% of stroke risk in the general population (137). Some other risk factors cannot be modifiable; however they contribute to definition of risk classes, such as older age, male sex and Hispanic or Black race. Actually also a low birth weight is associated in several populations with the risk of stroke later in life (136). Family history of cerebrovascular diseases could be a makeable factor for the predisposition to stroke, although the role of a genetic condition in the pathogenesis is still unclear (138).

In clinical practice, total risk to occur in a stroke event is categorized in high, intermediate and low risk. For instance patients who have experienced a stroke are supposed to be at high risk because they are highly likely to have a further stroke in the next 10 years. However, some asymptomatic patients with multiple risk factors, particularly those with type 2 diabetes, may also be considered as high risk (conventionally more than 20%) for future cerebrovascular events in the next 10 years. Most people with only a single risk factor are at lower short-term risk. Nonetheless, even single risk factor if severe and sustained, can lead to premature cerebrovascular events and should not be ignored in clinical practice (139). A number of tools for estimating risk of coronary heart disease or other atherosclerotic diseases have been developed over the past 10 years, including risk score charts, risk assessment algorithms and computer software programs (138).

The interaction of the various risk factors is not directly additive but more realistic a factorial event, and the risk to incur in death by a stroke attack increases with the number of factors (140). Certainly, modifications of life habits across wide populations are responsible for the emergence of most of these risk factors, and cultural modifications are common. These complications include increasing obesity and decreasing physical activity, conditions that are progressively much more important in these decades (138).

Risk factors have significant effects on the structure and function of blood vessels and also on their own interface with circulating blood. Many elements detailed previously alter vascular structure by promoting atherosclerosis and the solidification of the vessels walls. In brains these morphological changes are often associated with marked alterations in cerebral blood flow regulation. As a consequence, aging, hypertension, diabetes, and hypercholesterolemia impair vital adaptive mechanisms and the brain is not more adequately perfused (141). The ability of the endothelium to regulate microvascular flow is compromised, while the

increase in blood flow evoked by neural activity is suppressed, resulting in a mismatch between the brain's energy supply and demand (142).

Many of these cardiovascular risk factors increase production of ROS and promote inflammation in systemic and cerebral blood vessels. As shown in figure 28 the predominant vascular sources of ROS are the superoxide-producing enzyme NADPH oxidase, xanthine oxidase (XO), mitochondrial enzymes, and uncoupling of NOS, a state in which this enzyme generates superoxide instead of NO (143).

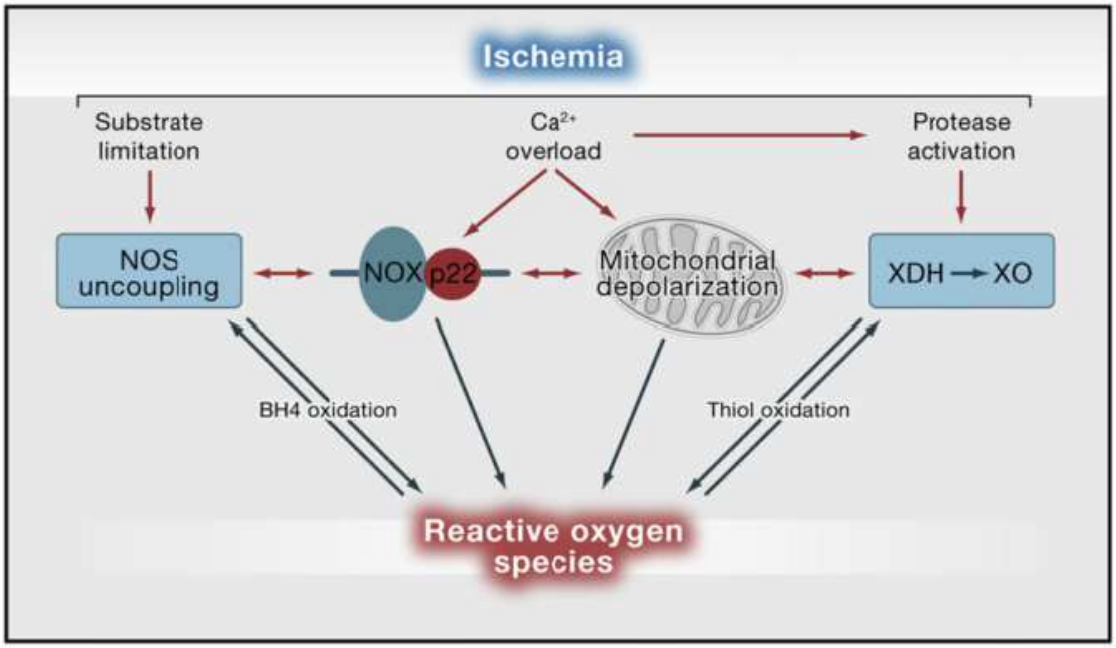


Figure 28. Predominant sources of ROS in brain and blood vessels. The increase of Ca^{2+} activates the superoxide-producing enzyme NADPH oxidase (NOX) and induced protease activation that is responsible for the conversion of xanthine dehydrogenase (XDH) to XO. Mitochondria become depolarized by the Ca^{2+} overload producing large amount of superoxide

Many of the damaging effects of oxidative stress on blood vessels are related to the biological inactivation of NO, thus loss of its regulatory effects leads to vasoconstriction and a reduction of vascular responses dependent by NO. This condition has implications on the regulation of microvascular flow (144). On the other hand, NO inactivation promotes key steps in vascular inflammation, such as platelet aggregation and leukocyte adhesion

to endothelial cells (145). In addition, ROS can also promote directly inflammation by increasing BBB permeability through up-regulation of vascular endothelial growth factor (VEGF) and by inducing the expression of cytokines and pro-inflammatory genes by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κb) activation (146). Considering that ROS may set the stage for inflammation, vascular inflammation, in turn, leads to ROS production, creating a vicious circle that enhances an increasing vascular damage.

Thus, vascular inflammation and oxidative stress are major pathways through which risk factors enforce their damaging effects on blood vessels. However, it remains to be determined how individual risk factors trigger the activation of one or both of these processes. This is a critical question for targeting preventive strategies in patients with specific risk factors. Exacerbation of vascular inflammation and activation of the coagulation cascade are likely to play a role. For example, the added vascular dysfunction and blood clotting abnormalities could precipitate vascular occlusion or hemodynamic insufficiency. This view is supported by the fact that acute stroke often occurs in the setting of increased circulating leukocytes and elevated plasma markers of systemic inflammation and vascular activation, which also predict a poor outcome (147).

1.1.3.TYPICAL NEUROPATOLOGY OUTCOME

Even though over the last two decades, advances have been made in diagnosis and treatment of acute stroke, the pathology is still a leading cause of death in developed countries. After an ischemic event complications include medical and neurological features. It is complicated define clearly which are the typical neuropathology outcomes after a stroke episode. Neurological complications are less frequent than medical complications but generally occur earlier in the course of stroke progression (148).

Principal clinical features observed are chest and urinary infections predominantly because of the hospitalization. Beyond these consequences an important tendency toward increased diagnosis of depression is reported after stroke (148). For what that concern neurological outcomes they are estimated to be the major cause of death in the first days (149).

The leading cause of death after stroke especially in the first week is brain edema. This complication occurs by the ionic imbalance due to energy depletion (150). It is possible to identify two types of edema after a stroke event, cytotoxic and vasogenic. The first one, not responsive to anti-edematous treatments, is characterized by the translocation of interstitial water into the intracellular compartment and occurs in first stages, when the BBB is still intact (151). After that, when the BBB is already compromised, the second kind of edema will be recognized. Vasogenic edema is identified by fluid movement from vascular to extravascular spaces. This process leads to an expansion of brain volume that means an increasing intracranial pressure, herniation, and reasonably additional ischemic injures (152). This feature is like to be more important and devastating for young patient than older, probably because of the age-related brain tissue atrophy that seems to have a role in the protection from edema formation (153).

Another important neurological outcome after ischemia is the hemorrhagic transformation of brain infarction. The main causes of

hemorrhagic conversion are the loss of microvascular integrity and disruption of neurovascular homeostasis (154). The mechanisms for the disruption are multifactorial, and these factors can interact with each other; they have been identified as treatment, inflammation, VGEF, NOS, and free radicals (151). Hemorrhagic transformation expands brain edema, leading to displacement and disruption of brain structures. The combination of these factors is associated with extremely high rates of mortality because increases intracranial pressure and induces apoptotic neuronal and glial cell death (155). This complication is more frequent in older people than in young patient owing to factors such as impaired rate of pharmacological treatment clearance and possible age-related microangiopathy (like cerebral amyloid angiopathy or hypertensive microangiopathy) (156).

Seizures can occur soon after the onset of ischemic stroke or can be delayed. Early seizures are usually defined as those that occur within 1 or 2 weeks after stroke and late seizures as those that occur after that. Although early seizures after stroke are thought to result from cellular biochemical dysfunction leading to electrically excitable tissue, late-onset seizures are thought to be caused by gliosis and the development of meningocerebral cicatrices (157).

Another common problem in the acute stroke settings is delirium. Delirium is defined as an acute transient disturbance of consciousness and a change in cognition with fluctuating intensity (158). The cause of stroke related delirium is far to be clearly understood, but changes in neurotransmitter concentrations (e.g., acetylcholine and dopamine, serotonin, norepinephrine, and GABA), a non-specific reaction to stress, and activation of the hypothalamic–pituitary–adrenal axis might have a crucial role (159).

Finally sleep disorders are frequent in the initial stages after stroke in the form of increased sleep needs (hypersomnia), excessive daytime sleepiness, or insomnia. Sleep-disordered breathing in patients presenting

with obstructive, central, or mixed apneas is common after stroke, occurring in about 50–72% of patients, and is both a risk factor and a consequence of stroke. The most common form of sleep-disordered breathing is obstructive sleep apnea, which is caused by cessation of nasal flow because of collapse of the upper airway (160).

Ultimately, recent studies support that patients with a history of stroke are associated with higher risk of AD compared with non-stroke patients (12). The pathologic characteristics of AD might be accelerated by cerebrovascular familiar predisposition or disease. Atherothrombotic stroke, the most common type of stroke, often reflects more severe cerebral atherosclerosis. The atherosclerotic lesions might alter endothelial permeability of the BBB, allowing greater exposure of parenchyma to systemically circulating molecules, including oxidants, cytokines, or β-amyloid protein. The presence of cerebrovascular injury, even if it does not cause measurable deficits itself, might cause increased cognitive dysfunctions in the presence of a concomitant degenerative process. In addition, if the strategic placement or a certain amount of brain tissue was injured by stroke, this might lessen the burden of AD pathologic changes required to produce symptoms of dementia. Association between stroke and AD has been reported in previous studies, but the literature on the opposite association (AD on stroke risk) is scarce (12).

1.1.4.CELL DEATH MECHANISMS IN CEREBRAL ISCHEMIA

A cerebral stroke event is characterized by a rapid decrease in the cerebral blood flow. From the moment that often this episode is a focal condition, is possible to see, in the central core regions of the insult, an almost total arrest of the blood flow. This area evolves rapidly to death, but surrounding this core, in the penumbra area, flow levels may fall below functional boundary yet transiently lie above the threshold of cell death.

The penumbra, a metastable zone, is considered the potentially recoverable tissue, because permits cell survival for a certain period of time (Fig. 29). This condition made penumbra the target for neuroprotective therapy (161).

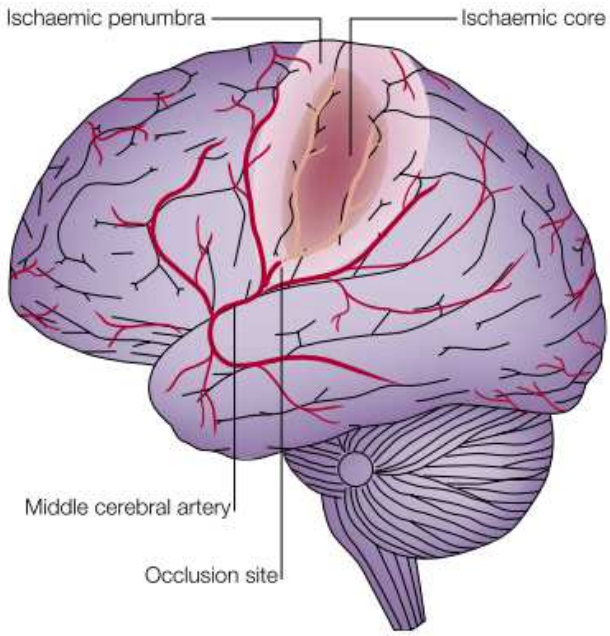


Figure 29. Penumbra and Core zone in the ischemic cerebral area.

The most upstream consequence of cerebral ischemia fundamentally is an energetic problem. In the area of reduced blood supply, ATP consumption continues despite insufficient synthesis, causing an increase of total ATP levels and a development of lactate acidosis with concomitant loss

of ionic homeostasis in neurons. In a second moment, the ischemic cascade follows involving multimodal and multicell downstream mechanisms.

The consistent loss of energy stores result in ionic imbalance, neurotransmitter release, and inhibition of reuptake. This is mostly important for the main excitotoxic neurotransmitter, glutamate. It binds to ionotropic receptors N-Methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA), to promote the influx of calcium in cells. When that happens the ion overload, triggers phospholipases and proteases to degrade essential membranes and proteins. Glutamate receptors are also responsible for an excessive promotion of sodium and water influx in the cell, producing cell swelling, edema and a consequent reduction of extracellular space (162). Not less important a massive calcium influx activates catabolic processes mediated by proteases, lipases, and nucleases. Because of the increasing calcium, sodium, and ADP levels in ischemic cells, the result is an excessive stimulation of mitochondrial oxygen radical production, within other sources of free radicals production such as prostaglandin synthesis and degradation of hypoxanthine.

ROS are directly responsible for lipid, protein, nucleic acid, and carbohydrate damage (163). From the moment that not only any corresponding up-regulation of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, but also other scavenging mechanisms are too slow to quenching ROS production, the effect of free radicals is especially toxic for cells. Thus, ischemic process and reperfusion activates a multimodal cascades will result in a complex mix of neuronal death comprising, necrosis, apoptosis, and autophagy.

Necrosis is a frequent outcome of ischemia and reperfusion that is characterized by cell and organelle swelling with subsequent rupture of surface membranes and the release of intracellular contents. This event is responsible for the immunoinflammatory response and cytokine production (164).

After acute ischemic cerebrovascular event, neurodegeneration in the perinfarct area is gradual and dominated by apoptosis that has been reported to be associated with an increased expression of p53 (165). Analysis of single nucleotide polymorphisms (SNPs) in chromosomal DNA showed that the Arg72 variant of p53 had a higher ability to induce neuronal apoptosis than the Pro72 variant and it is able to increase the vulnerability of neurons to ischemia-induced apoptosis through the mitochondrial pathway. Indeed, the Arg72 variant translocates to mitochondria where it binds and inactivates Bcl-XL inducing cyt C release and caspase-9 activation (Fig. 30). Caspase-9 activates downstream caspases and, among these, caspase-6 seems to be responsible for axonal degeneration and subsequent neuronal death. The time course of caspase-6 activation corresponds with axonal degeneration observed in human stroke as well as in other rodent models (166). Most importantly, the delayed time course of axonal caspase-6 activation, occurring between 12 and 24h, makes it an attractive molecular target for neuroprotection (167).

On the other hand, apoptosis may also be modulated by the transcription factor NF-kB during ischemia and reperfusion, because of limited oxygen availability is associated with its activation through a mechanism involving hypoxia-dependent inhibition of oxygen sensors (168). However, morphological evidence that ischemia causes classical apoptosis of neurons in the adult brain is less consistent. Outside of necrotic core of a typical brain infarct, there are neurons, in which condensation of chromatin is often an early result.

In contrast, cytoplasmic blebbing and the formation of typical apoptotic bodies are unusual, and nuclear DNA degradation is often delayed until morphological changes of cell death are advanced (169). Although there are many reports of apoptotic death of neurons in models of ischemic stroke in adult animals, relatively few of those have been based on detection of the characteristic morphological features of apoptosis (170).

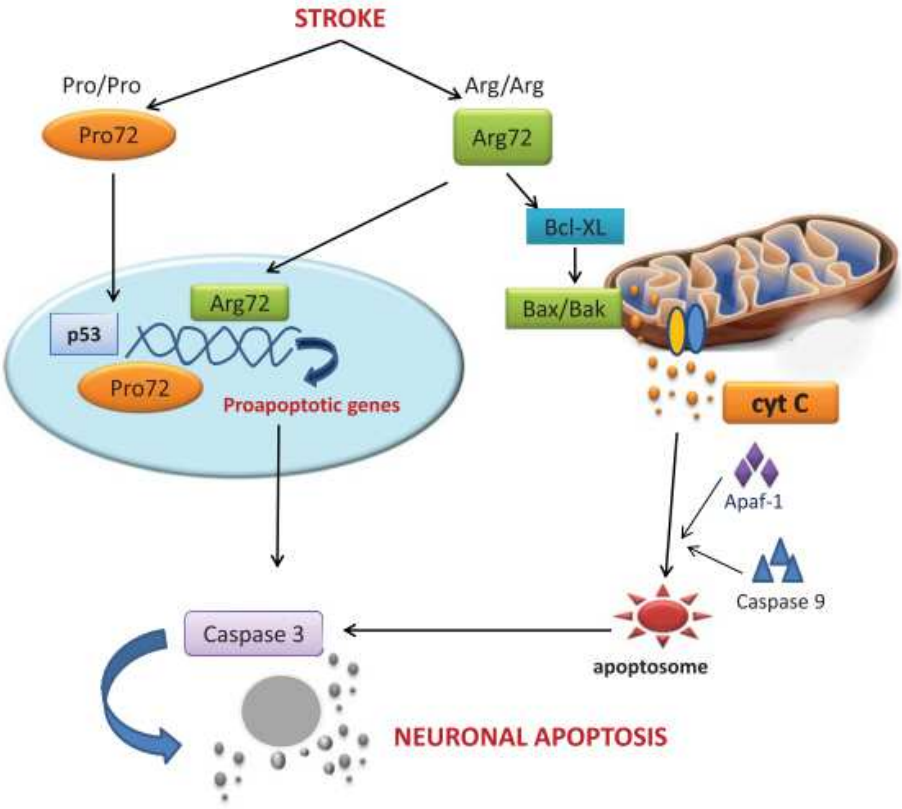


Figure 30. Apoptosis activation in stroke. Arg72 and Pro72 polymorphic variants can activate p53-downstream proapoptotic genes. Moreover, Arg72 increases the vulnerability of neurons to ischemia-induced apoptosis through the activation of the mitochondrial pathway: Arg72, but not Pro72, translocates to the mitochondria and directly binds to, and inactivates, Bcl-xL. This induces cyt C release, caspase-9 and -3 activation and finally neuronal apoptosis.

For this reason it has been suggested that 'caspase-mediated cell death' is a more accurate description than 'apoptosis' of the programmed cell death that can result from ischemia in the adult brain, especially because post-ischemic neuronal death may involve a combination of apoptotic and necrotic processes (171).

There is strong evidence supporting the idea that autophagy is an adaptive response to stress, such as nutrient deprivation, and the deletion of key autophagic genes accelerates rather than inhibits cell death (164). The hypoxia-inducible factor (HIF), a central mediator of hypoxic responses, also seems to regulate autophagy. The process of mitochondrial autophagy is induced by hypoxia and requires HIF-dependent expression of autophagic

genes, indicating a crucial role for HIF in the metabolic adaptation of hypoxic or ischemic tissues during conditions of limited oxygen. The first report of Nitatori and colleagues (171) demonstrated an increasing autophagy after cerebral ischemia, which showed that the increased lysosomes in neurons after transient global cerebral ischemia are mostly autolysosomes. Anyway, autophagy process is not always a protective outcome in cell life. In 2012 Shi and colleagues demonstrated that physiological levels of autophagy promote survival, whereas insufficient or excessive levels of autophagy promote death. It is possible that prolonged oxygen deprivation/reperfusion determines an excessive autophagy response, switching its role from protective to deteriorative (172). Probably the time at which autophagy is induced determines its role. Autophagy could play a protective role in ischemic preconditioning but have a different effect once ischemia/reperfusion has occurred (173).

In parallel with the molecular events briefly outlined here, another physiologic process called cortical spreading depression (CSD) has also been recently identified as a candidate target for stroke (161). CSD occurs when extracellular potassium exceeds a critical threshold; it consists in an intense depolarization of neuronal and glial membranes that slowly propagated by way of gray matter contiguity. The process is characterized by a near complete breakdown of ion gradients and near complete sustained depolarization that is express by an extreme shunt of neuronal membrane resistance with a consequent loss of electrical activity, neuronal swelling, and distortion of dendritic spines (174). Under physiological conditions, CSD is associated with a major cerebral blood flow rise in an attempt to match the increased metabolic demand. Under pathological conditions such as ischemia, can be observed an inverse hemodynamic response characterized by a blood flow reduction that increment that adverse event (175). In ischemia, these waves of CSD originating in the peri-infarct area can invade repeatedly the peri-ischemic tissue, adding a major metabolic demand on

penumbra, and at term, the spreading depolarization expands the volume of infarction (174) (Fig. 31).

This overview is useful to understand how is complicate define a specific way not only for the intervention but also in the prevention and in the definition of conditions necessary for a better outcome after an ischemic stroke event.

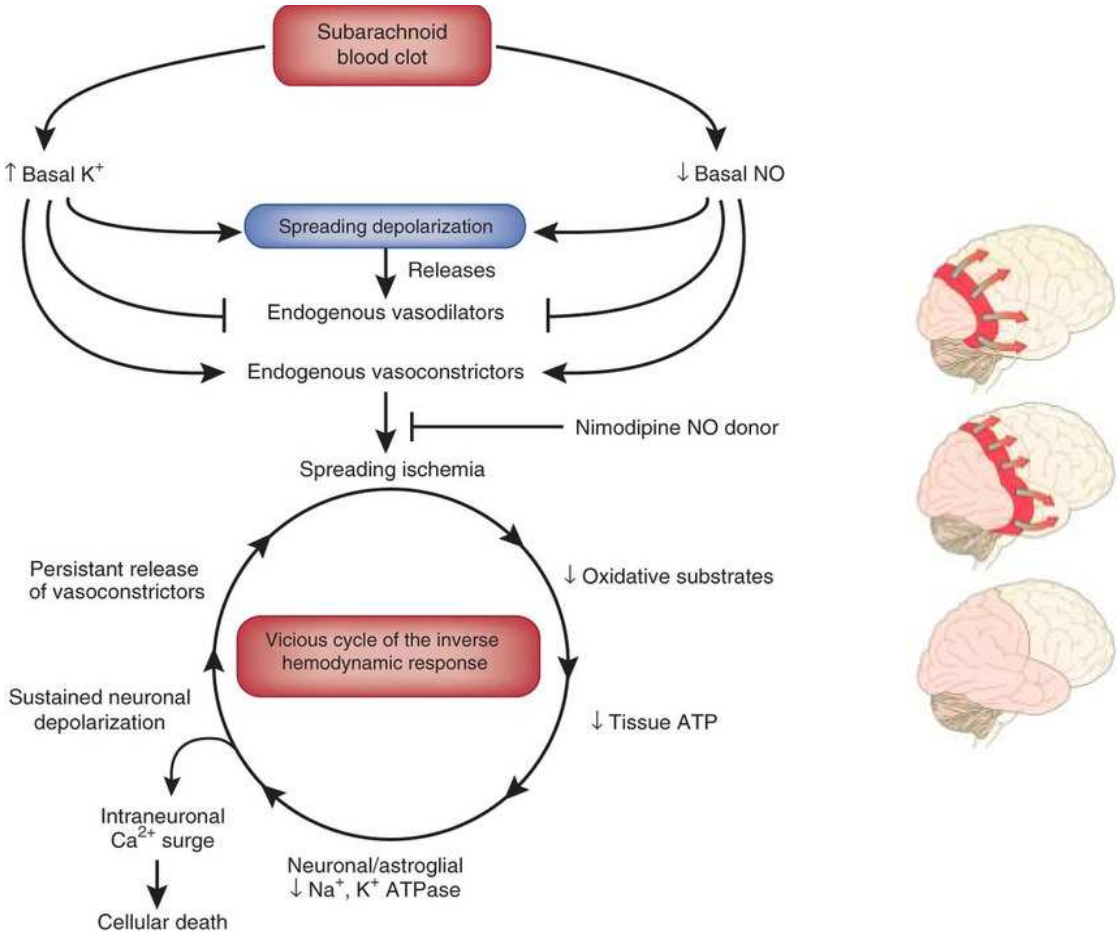


Figure 31. Cortical Spreading Depression (CSD). The vicious cycle underlying spreading ischemia. The decline in oxidative substrate supply while energy demand is increased, determines fall of tissue ATP with reduced sodium pump activity. There is also failure of neuronal repolarization and, therefore, continued release of vasoconstrictors, which maintains the perfusion deficit while the perfusion deficit maintains the depolarization.

1.2. ANIMAL MODELS

Animal models of stroke can contribute to understand etiology of different subtypes of stroke so as develop a better prevention and treatment strategies. We have to consider that for some pathology many animal models are not able to represent the whole disease process but aim to enable detailed study of specific aspects. Animal models are also useful in testing promising therapeutics interventions, both pharmacological and non-pharmacological. Most animal models of stroke are rodents, rabbit, pigs, dogs, and in few cases primates. These models can be divided in two types: models in which stroke are induced through artificial ways and animals in which stroke occur spontaneously (176).

The choice of the model depends on the research question. Acute stroke presents inherently a high variable clinical condition that may be eliminated by animal models, but some of them can fully mimic the complexity of the stroke event. Before choosing an appropriate model there are few important key points to establish. First, consider for which types of human stroke the treatment under investigation may be relevant, then lesions procedures must be reproducible and minimal invasive. Not less important in the choice of the model used is if it allows assessment of a relevant, functional, neurobehavioral outcome (177).

1.2.1.INDUCED STROKE MODELS

Models of global ischemia

Acute models of global ischemia may mimic the cerebral damage that occurs after cardiac arrest, while chronic cerebral hypoperfusion models are representative of subcortical white matter damage similar to what is thought to be due to cerebral small vessels disease. Often the damage is induced in rodents by permanent occlusion of both vertebral arteries and or transient ligation of two common carotid arteries. These methods are responsible of an extensive bilateral forebrain injury (178). In order to produce mainly white matter lesions, it is possible to use a chronic global hypoperfusion model either by ligation of common carotid arteries or stenosis using external microcoils (179) (Tab. II).

Table II. Rodent models of global ischemia

MODEL	ADVANTAGES	DISADVANTAGES
ACUTE		
Four-vessel occlusion	Reversible forebrain ischemia, may be induced in awake animals	Two-stage surgery
Two-vessel occlusion	Reversible forebrain ischemia, One-stage surgery	Necessity of systemic hypotension
Asphyxia cardiac arrest	Whole brain ischemia	Intensive postsurgery care
CHRONIC		
Bilateral common carotid artery ligation	Produces white matter changes similar to leukoaraiosis	High death rate
Bilateral common carotid artery stenosis	Produced milder reduction in cerebral blood flow	Take long time to develop lesions
Asphyxia cardiac arrest	Whole brain ischemia	Intensive postsurgery care

Models of Middle Cerebral Artery occlusion (MCAo)

Models of MCAo were developed to mimic the consequence of the most frequent ischemic stroke. This model has been reported to induce long-term sensorimotor and cognitive deficits as well as impairments of postural and sensory reflexes (180). In order to produce vessel occlusion endovascular or surgical procedures are commonly used. In this way it is possible to perform a permanent or transient event. A model of transient MCAo permits to investigate either brain injury related to the ischemic event and the consequences of reperfusion (181). Another way to induce MCA is thromboembolic occlusion that is commonly obtained by injection of blood clots. This model permits to investigate the potential of antithrombotic drugs (182). It is possible to induce a thromboembolic stroke also by injection of sodium dodecyl sulfate (SDS) detergent or by deliver of photoactivated thrombogenic agents (183, 184) (Tab. III).

Table III. Rodent models of focal ischemia

MODEL	ADVANTAGES	DISADVANTAGES
Endovascular MCAO	Most common method used for permanent or transient stroke	Risk of vessel rupture
Surgical MCAO	Control of occlusion site, less variability	Necessity of craniotomy
Thromboembolic MCAO	Mimics most common cause of ischemic stroke in human	Higher variability
Phototrombosis	Less invasive procedures	Less relevant for human condition
Intracarotid injection of SDS detergent	Selective perforating artery occlusion	Unpredictable distribution of infacts

Models of induced sub-arachnoid hemorrhage (SAH)

Models of SAH present intracranical bleeds in the space between the arachnoid membrane and the pia mater, frequently by endovascular perforation or by injection of blood. The second one allows a close control of

the hemorrhage volume, but the first one describes in a better way what happen in human pathology (185) (Tab. IV).

Table IV. Rodents model of sub-arachnoid hemorrhage		
MODEL	ADVANTAGES	DISADVANTAGES
Endovascular perforation	Reproduce aneurysmal clinical condition in humans	High mortality rate
Intracisternal blood injection	Severity may be controlled by blood quantity and number of injections	Nonphysiological blood distribution

Models of induced intracerebral hemorrhage

The most used model to investigate intracerebral hemorrhage consists in the injection of bacterial collagenase or blood in a specific brain area by a stereotaxic probe. From the moment that collagenase disrupts the basal lamina of vessels causing a spontaneous bleeding that permits to investigate hemostatic aspects of the pathological event (186) (Tab. V).

Table V. Rodents models of intracerebral hemorrhage		
MODEL	ADVANTAGES	DISADVANTAGES
Intracerebral injection of bacterial collagenase	Mimics humans condition	Bacterial collagenase may induce inflammatory response
Intracerebral blood injection	Without confounding inflammation	Neurofunctional deficits resolve more rapidly

1.2.2.TRANSGENIC MOUSE MODELS

A number of rare forms of stroke occur in humans as a result of single gene disorders (187). This includes conditions responsible of specific vasculopathies that may give lacunar ischemic strokes. The most common is cerebral autosomal dominant arteriopathy with subcortical infarcts and leuco-encephalopathy (CADASIL), resulting from mutations in the Notch3 gene. In this regard, several Notch3 mutant mouse models have been developed, which reproduce the features of human CADASIL vascular pathology accompanied by progressive white matter damage, without infarcts (188). These and other transgenic mouse models are a developing study area because permit the investigation either into the disease mechanism of rare forms and new treatments for the uncommon monogenic subtypes of stroke.

It is important underlie the presence of significant animal models of stroke that permit to investigate single mechanism of interest, such as hypertension or inflammation. In this context it is possible to study the involvement of a single component in the outcome of the pathology that allows identifying a probable strategy for neuroprotection.

1.3. STRATEGIES OF NEUROPROTECTION

Neuroprotection defines any strategy with the aim of antagonizing molecular and cellular events responsible for the ischemic damage, allowing neurons to survive when cerebral blood flow is reduced and to stabilize penumbra area (189). One approach to manage stroke injury is known as preconditioning. Preconditioning is a well-defined phenomenon by which is possible to give protection and tolerance by a small harmful stimulus before a damaging event (190). Preconditioning induces a transient window of protection that requires gene activation and new protein synthesis (191). If stroke occurs during this window after preconditioning, the response is reprogrammed to produce new signaling cascades to promote protection and resist injury. Preconditioning can be viewed in three sequential phases: a priming phase that sets up protection, a refractive phase when the system is resistant to injury, and a neuroprotective phase that is characterized by a reprogrammed response to stroke that reduces injury (190). Obviously the preconditioning process results to be useful in research studies because allow to identify which mechanism may be modulate to target an efficient alternative neuroprotective therapy. On the other hand, for what that concern clinical practice, application of preconditioning phenomenon is not frequently helpful.

We have already explained how not only the ischemic process, but also the reperfusion phase is fundamental in the production of ROS and the subsequent cellular death cascade. In this instance appear clear that a neuroprotective approach focused on the antioxidant responses would be the powerful way to prevent, slow down and control consequences of a cerebral stroke and reperfusion injuries.

Because of the complexity of various mechanisms responsible of the tissue damage after stroke, the best approach has to suppress many of them, alone or by a combination.

The production of ROS and other free radicals is a consequence of inflammation but also of excitotoxicity (192). These molecules, such as $^{\bullet}OH$, O_2^{2-} and peroxynitrite are highly reactive species, able to damage multiple cellular components, leading to cell death. One way of reducing oxidative stress is to decrease the production of free radicals. For instance, although NO is a normal signaling molecule in the body and has beneficial effect in stroke, larger amounts resulting from increased activity of the induced nitric oxide synthase (iNOS) can lead to aberrant signaling or react with superoxide to produce peroxynitrite. At this purpose the administration of an antioxidant, able to decrease iNOS production and increases expression of endothelial nitric oxide synthase (eNOS), may be efficient for the reduction of histopathological changes. Another source of ROS is the NADPH oxidases, so inhibitors of these enzymes could be beneficial as the induction of SOD and GPx (193). In this context it is important to remember that many exogenous compounds, natural or synthesis origin may play a crucial role to reduce ROS production and to contain damage related.

In a stroke event, disruption of the BBB is commonly associated with the action of two matrix metalloproteinases, MMP-2 and -9. These metalloproteinases are constitutively expressed at low levels, however ischemia increases them expression and activity. Other factors are involved in BBB permeability after stroke, including the extent of tight junction formation between endothelial cells and the effects of treatment with tissue plasminogen activator. The activity of MMPs is regulated endogenously by the tissue inhibitor of matrix metalloproteinase, to this end treatments that stimulate it may show a significant neuroprotective role against brain damage induced by stroke (193).

During stroke, depletion of neuronal oxygen and energy reserves leads to the release of toxic amount of the neurotransmitter glutamate into the extracellular space. Glutamate excitotoxicity plays a significant role in the pathology, for this reason a valuable key for neuroprotection is to diminish

glutamate release or manage the action of glutamate receptors in the brain (125).

We have already explained that apoptotic process is fundamental in the evolution of tissue damage after stroke. The crucial role of apoptosis is also related to the fact that this cell death pathway is characteristic of penumbra area, the part of lesion where is possible to reverse neuronal loss advancement. Apoptosis may be induced by mitochondrial pathway or caspase dependent pathway. For this reason reduction of activated caspase-3 levels may be the goal for neuroprotective treatments (193).

Autophagy appears to have a dual role in the response to cellular damage, absorbing damaged components as a protective measure in some cells and serving as a mechanism of cell death in others (194). Induction of autophagy prevents cell death by apoptosis and in this context is considered to be beneficial. Alternatively, the inhibition of autophagy can also be considered neuroprotective. In pMCAO rats, ischemic postconditioning inhibited the induction of autophagy and reduced infarct size and edema (195). It is hopeful that further research will determine if the effect of autophagy in stroke would be beneficial or harmful.

Neuroprotection will be the fundamental key point for a good outcome after a stroke event, all mechanisms listed before are considered potential target for a powerful neuroprotective therapy.

1.3.1. INFLAMMATION INVOLVMENT IN ISCHEMIA PROCESS

Another important factor involved in the ischemia process is neuroinflammation. Inflammation is a double faced mechanism, on one hand it determines an increased cerebral blood flow to the affected area and the removal of damaged tissue by phagocytic cells; but on the other hand secretory factors released by cytokines and chemokines may reach toxic levels, besides the incremented production of ROS and the consequent destruction of the BBB (196).

In an intact brain, the trafficking of cellular and molecular components from peripheral circulation is regulated by the BBB. In this condition, immune response in the CNS is regulated by resident microglia. Following a brain insult, the tight junctions between endothelial cells become permeable, allowing peripheral immune cells to infiltrate brain parenchyma (197). Acute inflammation after stroke originates from activation of resident immune cells, the microglia, followed by infiltration of peripheral inflammatory cells (198). Microglia exists in two different states, the M1 and the M2. On activation microglia takes on the M1 phenotype and secretes various proinflammatory molecules including interleukin (IL)-1β, TNF-α and ROS. When microglia turns on the M2 phenotype, secretes the anti-inflammatory molecules such as IL-10 (199). Macrophages, which reside in the perivascular space, are a large driving force behind infiltration of peripheral immune cells into the brain parenchyma (200). Macrophages exist in multiple states that are phenotypically and functionally distinct, such as microglia phenotypes, M1 and M2. After macrophage and neutrophil infiltration, lymphocytes begin to infiltrate the brain as well. Under normal conditions, T cells are not able to migrate through the BBB and enter the brain. However, after injury, activated $CD4^+$ and $CD8^+$ T cells can enter in the brain (201). In addition, there are CNS-specific T cells that promotes leukocyte passage into the CNS after injury (199). Approximately 15% of these T cells are $CD4^+$ TH1 cells, which stimulate cytotoxic $CD8^+$ T cells, and secrete a membrane-permeabilizing

molecule. Furthermore they induce apoptosis through caspase activation or by activation of the Fas ligand pathway. In addition to TH1 cells, TH2 cells secrete numerous interleukins (IL-4, 5, 9, 10, and 13) that are important for activation and recruitment of cells, namely B cells, involved in the humoral immune response (197).

ROS and inflammatory mediators cause endothelial cell and leukocyte expression of adhesion molecules, promoting the adhesion and migration of circulating leukocytes that leads to a rapid inflammatory state at the site of injury. Furthermore these leukocytes in turn release inflammatory cytokines that lead to tissue damage in the core and the ischemic penumbra (197) (Fig. 32).

Inflammatory response has a crucial role in the tissue damage of a stroke event. TNF-α interacts with two receptors, R1 and R2, that mediate death signals by the Fas associated death domain (FADD) and inflammation through the NF-κB, respectively. On the other hand, the interleukins are another important set of molecules in the process of inflammation. IL-1 is proinflammatory, and IL-6 has both pro- and anti-inflammatory effects (202).

As one of the early initiators of inflammation after stroke, TNFα is an excellent target for neuroprotective treatments. Activation of NF-κB by TNFα initiates a signaling cascade that regulates a number of inflammatory processes, making it a good point of intervention. The various signaling cascades induced by stroke lead to the activation and recruitment of inflammatory cells to the site of injury.

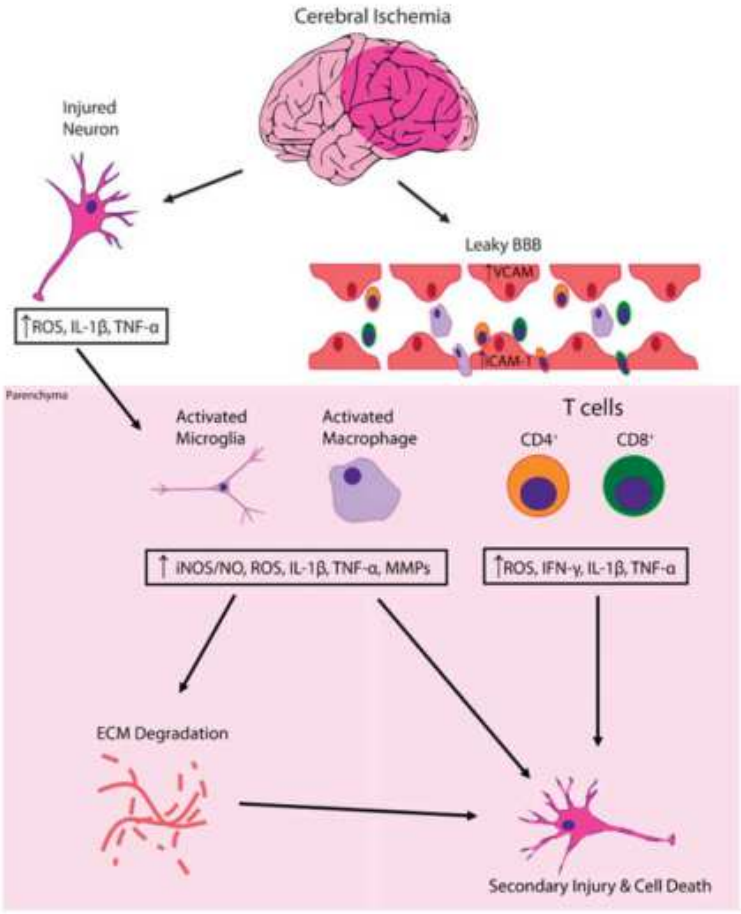


Figure 32. Inflammatory cascade. Cerebral ischemia leads to an initial cell necrosis and generation of ROS and proinflammatory molecules like IL-1β and TNF-α from injured neurons as well as inducing BBB dysfunction with loosening tight junctions between endothelial cells. This causes extravasation of circulating leukocytes into the brain parenchyma. When in the brain, leukocytes and activated microglia generate a variety of proinflammatory molecules and continue to generate ROS. These events lead to secondary brain injury, increased inflammation, and ultimately cell death.

In the early stages of stroke, prior to the infiltration of neutrophils and macrophages from other locations, resident microglia is the primary inflammatory cells in the brain. Microglia continues to be involved well into long term recovery and it has been observed 28 days following stroke in MCAo rats (202). Although microglia serves a beneficial purpose by removing dead tissue, it also releases secretory factors that can accumulate to toxic levels, particularly in cases of excess activation such as stroke. The processes involved in inflammation may not only directly contribute to brain

damage following stroke but may also activate secondary mechanisms that lead to further damage. The activity of large numbers of inflammatory cells in the affected area, combined with low oxygen and ATP levels, leads to the formation of ROS and the onset of oxidative stress (193).

Neuroinflammation after ischemic stroke is, for the most part, a self-limiting event. Resolution of inflammation in the brain seems to be an active process in which inflammatory mediators are suppressed by regulatory mechanisms (193). Furthermore, resolution of inflammation in the brain, involves clearing of dead and dying cells primarily by the phagocytic activity of activated microglia with the help from infiltrating macrophages as well as production of antiinflammatory, proregenerative signaling molecules which suppress inflammation and exert neuroprotective effects on the surviving cells. In the chronic phase of stroke, there is little contribution from either resident or peripheral immune cells; however, there is still an increase in the number of microglia that help in brain repair by clearing of dead and dying cells (192). By understanding how inflammation increases the susceptibility toward premature CSN death, we can better design therapies to promote brain repair (193).

1.3.2. THE ROLE OF CD36 RECEPTOR IN INFLAMMATORY RESPONSE

CD36 is a class B scavenger receptor that regulates physiological and pathological functions. This receptor is an 88 kDa glycoprotein that consists of two transmembrane domains, a large extracellular domain, and two short cytoplasmatic tails. Extracellular domain includes a hydrophobic region that may be buried within the plasma membrane, a proline-cysteine rich domain and ten potential N-linked glycosylation sites (203) (Fig. 33).

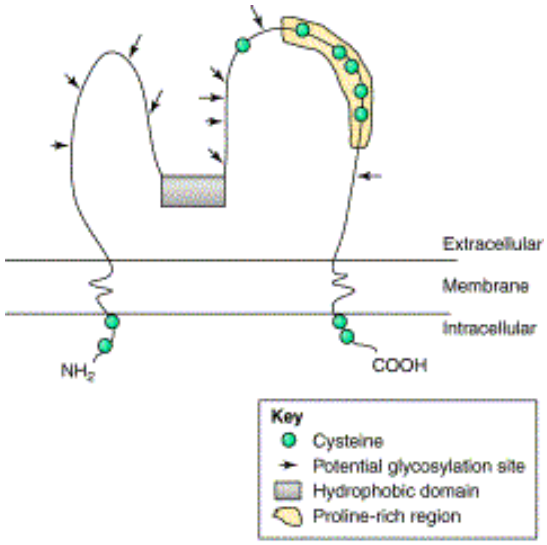


Figure 33. Structure of CD36 receptor

CD36 is expressed in many types of cells, from microvascular endothelial cells to microglia, macrophages, platelets, and adipocytes. For this reason this receptor is involved in numerous processes including inflammatory response. It is important to underlie that activation of CD36 receptor is involved in many stroke risk factors, like hypertension, dyslipidemia and diabetes. This consideration makes the receptor of interest an important key point in neuroprotection strategy not only in stroke pathology but also in neurodegenerative diseases where BBB integrity and cerebrovascular abnormalities have a fundamental role, such as AD and atherosclerosis.

As the most primordial function in the organism, innate immunity serves to recognize modified or oxidized phosphatidylcholine/serine of apoptotic cells (204). Triggered by tissue damage the innate immune system mediates inflammatory responses through a family of pattern recognition receptors. The receptors include toll-like receptors (TLRs) and CD36 which may recognize endogenous danger signals from damaged tissues (205). The pathogenic roles of CD36 and TLR2 in cerebral ischemia have been independently reported. CD36 expression increases in the post-ischemic brain, and CD36-knockout mice present a profound attenuation in the molecular and cellular inflammatory response induced by focal cerebral ischemia (206). Similarly, TLR2 expression is increased during ischemia and its deletion results in reduced ischemic injury (207). The closely shared function and physical association between CD36 and TLRs suggest that CD36 is involved in innate immune responses following cerebral ischemia. Targeting CD36 in an attempt to attenuate innate immune responses under pathological conditions may be a potential strategy for stroke therapy. Of particular note is that clearance of apoptotic cell bodies by phagocytosis, an essential function of scavenger receptors, may be an important process for tissue remodeling after injury (208).

In particular, CD36 recognizes pathogen-associated molecular patterns and induces an inflammatory response through activation of NF-κB (209). In cerebral ischemia, CD36 mediates free radical generation and contributes to tissue injury in the postischemic brain (210). The pro-inflammatory responses activated through interaction between CD36/CD36 complex and ligands are associated with generation of free radicals, cytokines, chemokines and foam cells, making this as an integral part of many pathological processes. In conclusion, neuroprotective strategies may be aimed at attenuating injury-induced inflammation by targeting CD36.

2. AIM OF THE STUDY

Stroke is the third leading cause of death worldwide and the main cause of permanent adult disablement in industrialized countries (124).

In USA strokes hit every 40 seconds and cause death every 4 minutes. Among survivors, the capacity of work is compromised in 70% of patients and 30% of them need assistance with self-care. Clearly appears how the stroke pathology is a really massive and economic problem worldwide (127).

Strategies of interventions are aimed to restore cerebral blood flow, but there are no efficient therapies for the damage induced in tissues hit by the cerebral ischemia or by the reperfusion phase.

For what that concern prevention of the disease, this pathology may be directed from many different risk factors, principally related to cardiovascular pathologies thus, the most efficient way to reduce the incidence is their modulation, and the education on a healthier life style. For this reason, patients considerate at risk are treated with antihypertensive drugs or statins.

The role of neuroinflammation is considered more and more important in the outcome of stroke. Neuroinflammation is involved in neurodegenerative diseases, both chronic and acute, and this underlines its crucial role and a connection point among various pathologies affecting CNS.

CD36 is a class B scavenger receptor that regulates physiological and pathological functions. For this reason, it is expressed in many types of cells, making the receptor involved in numerous stroke risk factors, where the inflammatory response shows a crucial role.

The aim of this work was to elucidate the potential neuroprotective role of a negative modulation of CD36 receptor, in a long term study, after MCAo.

For this purpose, we used an integrated approach of behavioral tests, motor and cognitive, and immunohistochemistry evaluation.

For the behavioral analysis we have used an extended variety of motor tests to evaluate functional, strength, balance, coordination and the extension of the lesion. This was possible by Modified Bederson test, Hanging wire test, Pole test, Corner test and Sticky tape test. For what that concern cognitive evaluation, we investigated if the declarative and non-declarative memories were compromised through Novel Object recognition test and the Y-maze test from 3 weeks after stroke.

Finally, after the sacrifice, we utilized immunohistochemistry in fluorescence to evaluate the extension of ischemic damage, microglia and astrocyte activation, neurogenesis and angiogenesis processes.

3. MATERIALS AND METHODS

3.1. ANIMALS

Male mice C57BL/6JOlaHsd (Jackson Laboratories, USA) and CD36$^{-/-}$ (8 weeks old, 20 g body weight at the beginning of the experiment) were housed 5 for cage under 12 h light/12 h dark cycle (lights on from 6.00 a.m. to 6.00 p.m.) with free access to food and water in a temperature (22±2°C) and humidity (60%) controlled room. Experimental procedures were carried out in the light cycle (from 9.00 a.m. to 5.00 p.m.) including control groups in each test performed. In particular we used four experimental groups; wild type and CD36-knockout operated and sham, both wild type and CD36-knockout. During six weeks after the lesion, once a week, we have assessed the extent of the lesion using several motor e cognitive tests. In order to acclimatize animals at the new environment, mice were moved in the behavioral room for at least 1 hour before starting the tests. At the end of behavioral analysis, mice were sacrificed by perfusion to perform immunohistochemistry.

3.1.1. TRANSIENT OCCLUSION OF THE MIDDLE CEREBRAL ARTERY

Animals were anesthetized using 4% isoflurane in oxygen, and anesthesia was maintained with 1.5-2% isoflurane in an oxygen/room air mix. Mice are kept at 37±0.5°C during the surgical procedure using a heated surgical surface. Using instant krazy glue and accelerator, the Doppler fiberoptic probe is attached to the parietal bone 2 mm posterior and 5 mm lateral to bregma. The fiberoptic probe is then connected to laser Doppler flowmeter and cortical perfusion flow recorded. At this position, was detected a reduction >85% in cortical cerebral blood flow

upon occlusion of the middle cerebral artery (MCAo) on the right side, resulting in a reproducible cerebral infarct. Ischemic lesion was inducted by the introduction of an intraluminal filament to occlude MCA for 30 minutes (Fig. 34).

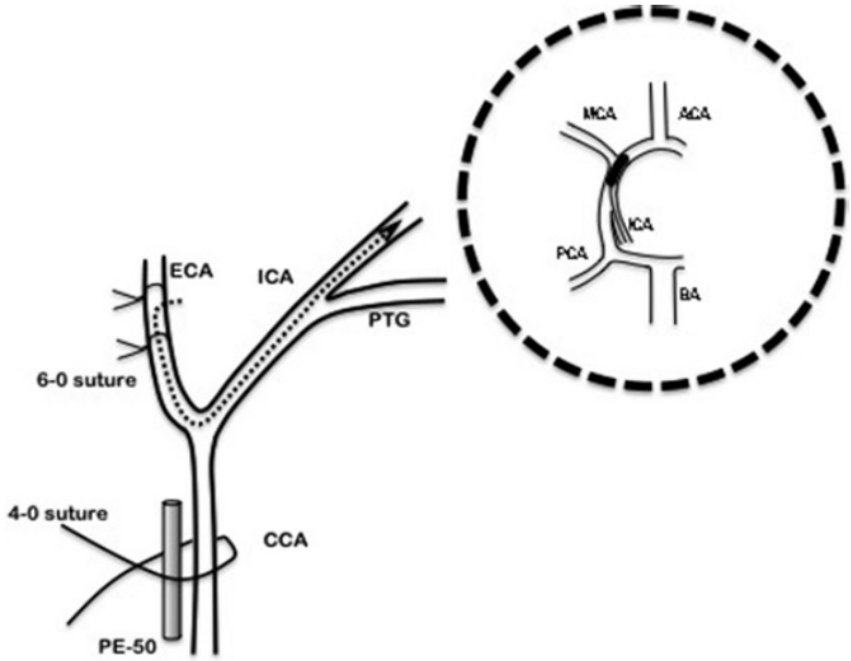


Figure 34. Intraluminal filament MCAO. The filament is inserted between the two external carotid artery (ECA) 6-0 suture knots, and advanced along the internal carotid artery (ICA) until it reaches the origin of the MCA.

After 30 minutes, the filament is retracted and an 80% recovery of cerebral blood flow must be observed in reperfusion that has to be 30 minutes long. After the surgery animals were maintained in a temperature controlled cage until it regains complete consciousness (211).

Motor behavioral assessment was performed by day 4 after the surgery and then once a week for six weeks. Cognitive evaluation was performed by the third week until the sacrifice.

3.1.2.TEMPERATURE

The temperature was measured every day of the behavioral test to verify the condition of health after the induced cerebral ischemia.

3.1.3.WEIGHT

Animals were weight every day of the test to evaluate the condition of health and the percentage loss of weight.

3.1.4.MODIFIED BEDERSON TEST

Modified Bederson test allows to defining neurological assessment after MCAo surgery. Mice were scored from 0 to 4 according with the criteria showed in table VI (212):

Table VI. Modified Bederson test scoring	
0	normal motor function
1	flexion of the torso and forelimb contralateral to MCAo when mouse is suspended by tail
2	circling to the side contralateral to MCAo, when mouse is left on a flat surface
3	leaning to the contralateral side at rest
4	no spontaneous motor function

3.1.5.HANGING - WIRE TEST

Hanging-wire test was performed to evaluate forepaw strength. Animals are made to grasp, using their forepaws, the middle of a wire that is about 30 cm long and suspended at 50 cm above a soft surface. To prevent that mice could reach the pole that suspends the wire, poles are covered with a white paper (Fig. 35).

METHOD

Two days before the surgery, mice started the training for two days. The first day of habituation to the tool is made through positioning animals on the wire just one time. The day after each mouse is collocated on the wire for one minute. If the animal can not stay on until the end of the minute, it is placed again until the end of the time. The day of the test, animals performed three trials with a cut off at one minute.

Values were expressed as mean of three trials for each experimental group.

Figure 35. Hanging wire test. Mice were left on the wire allowed to use only forepaws.

3.1.6.STICKY TAPE TEST

Sticky tape test was performed to evaluate sensitivity to perceive unknown objects and the ability to control faint movements. Briefly, the test consists in the evaluation of the time that animals need to remove from forepaws two little tapes earlier applied (Fig. 36). In this way it is possible toestablish not only the time to remove the tape, but also differences in the perception between ipsilateral and contralateral sides. Furthermore latency in the reaction from the time of the first contact with

the tape and the moment of removal can be an indication for the control of faint movements.

METHOD

The day before the surgery animals are trained in order to exclude mice with a side preference. Tapes of two different colors having dimension of 4 mm of length and 3 mm of height are prepared. Animals are located in an empty cage for one minute for the habituation at the new environment. Then a little piece of tape is applied on the forepaws, with attention to use pieces of the same dimensions and in the same position on each paw. After that, mice are left in the cage and times needed to touch for the first time and remove tapes on the right and left are recorded. The training consists in five trials or less, if mice are able to carry out the task in fewer than 20 seconds. The day of the test animals carried out one trial with a cut off at 3 minutes.

Values were expressed as mean of the time in seconds and as relation between contralateral and ipsilateral sides.

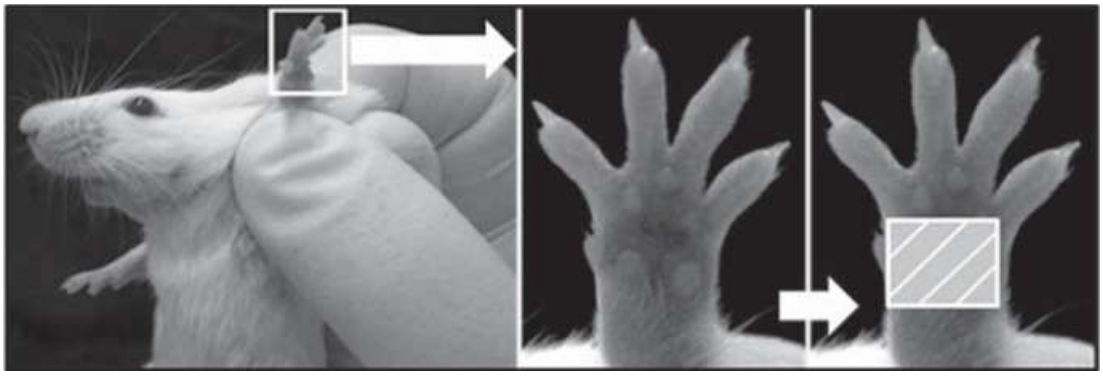


Figure 36. Sticky tape test. Two small pieces of colored tape with same dimensions were attached to the forepaws using the same pressure.

3.1.7.CORNER TEST

Corner test was performed to evaluate sensory motor integration. Briefly, animals were placed between two boards angled at 30° with a small opening along the joint between those to encourage entry into the corner (Fig. 37). As the mouse enters the corner, both sides of the vibrissae are stimulated causing the mouse to rear on its hindlimbs and turn to the opposite side. Normal mice not subject at ischemia have on average a 50% chance of turning to each side. However, mice subjected to cerebral ischemia have a >50% chance of turning to the right.

METHODS

A total of ten trials are performed with a cut off at 10 minutes. The direction that the mouse turns is recorded and the total percentage turns in the right (ipsilateral at the lesion) direction is calculated.

Values are expressed as percentage of the ipsilateral rotation on the total number of turns.

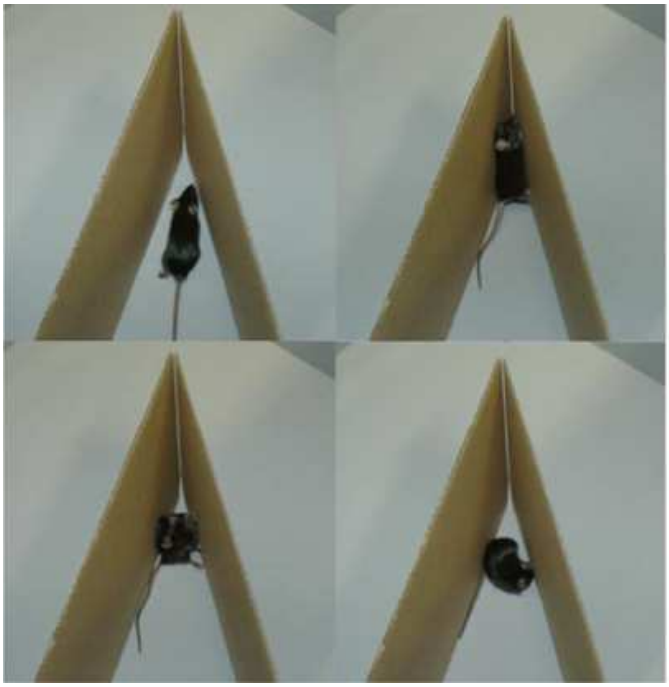


Figure 37. Corner test. When mouse entered in the corner it can fell with the vibrissae the two boards and turned on the right (ipsilateral) or left (contralateral) side.

3.1.8.POLE TEST

The Pole test was first used to study motor involvement in PD. The test allows evaluating coordination and the balance of mice. Briefly mice were located on a pole with a diameter of about 1 cm and height 50 cm. The pole may be made of wood or any material that present a rough surface to allow animals walk over it. Mice were placed on the top of the pole facing up. The test consists in the evaluation of the time needed by the animal to turn around itself (time turning), and the time to reach the floor (time descending) (Fig. 38).

METHODS

Two days before the surgery, mice start the training for two days. The first day animals were placed on the pole facing down for five trials. Once the mice reach the floor, they are left to explore the environment for one minute. The day after, animals were located on the pole again, but this time, facing up. The time to turn around and the time to reach the floor are recorded for five trials, or less if animals took fewer than 10 seconds to carry out the task. The day of the test animals are placed on the pole facing up one time with a cut off at 1 minute.

Values were expressed as time in seconds related to the best time reach in the training session.

Figure 38. Pole test. Mice were located on the pole facing up, the task consists in turning around to be facing down and descending the pole to reach the floor.

3.1.9.NOVEL OBJECT RECOGNITION TEST

The Novel Object Recognition test was carried out to evaluate the involvement of subcortical structures responsible of the construction of the declarative memory after MCAo (Fig. 39).

METHODS

The day before the test animals were trained for the habituation at the new environment. Mice were placed in an empty clear box of 50x30x30 cm dimensions and left to explore the environment for ten minutes. The day of the test, each mouse was submitted for the training session. Animals were located in the box for five minutes with two similar objects in color and shape. After a retention time of 30 minutes, mice were placed again in the box for five minutes, but this time with one of the two object was replaced by one completely different in color and shape. The experiment was recorded by video camera located over the box. In order to evaluate the time spent in the exploration of each objects, registrations obtained were analyzed by Any-Maze program.

Values were expressed as the percentage of time spent in the exploration of the new object related with the total time spent to explore both objects.

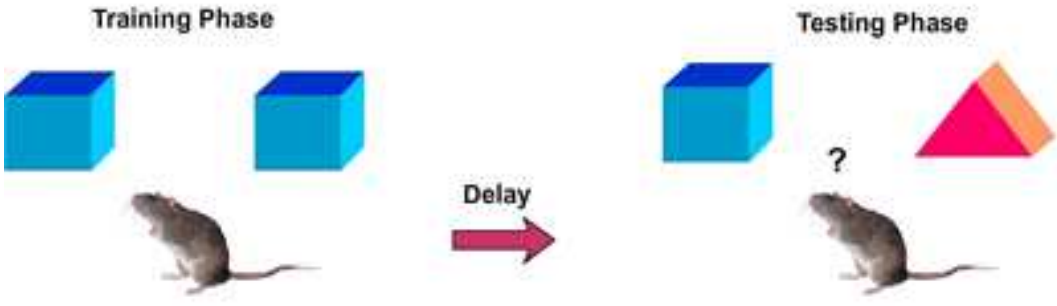


Figure .39. Novel Object Recognition test. After a training phase of five minutes with two similar objects, mice are placed in the box with two different objects for five minutes.

3.1.10. Y-MAZE TEST

Y-Maze test was carried out to evaluate the involvement of subcortical structures responsible of the construction of the non-declarative memory after MCAo (Fig. 40).

METHODS

The maze utilized for the test presented three similar arms with an angle of 120° between each other. Animals were always located in the same arm of the maze. Mice were submitted at a training session of five minutes with one arm of the maze closed by a door. After 30 minutes of retention animals were placed again in the maze for five minutes, but allowed to explore all arms of the apparatus.

Values were expressed as percentage of the time spent in the exploration of the new arm related with the time spent in the exploration of all three arms.

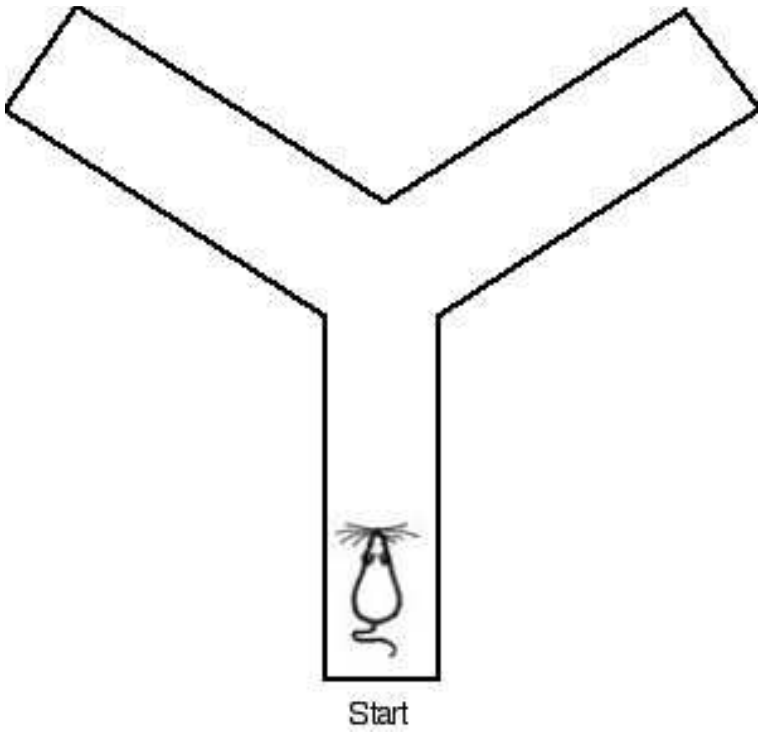


Figure 40. Y-Maze test. After a training phase of five minutes with one arm closed, animals were left to explore freely the maze for five minutes.

3.1.11. SACRIFICE AND TISSUE PREPARATION

Six weeks after the surgery, when behavioral analysis was completed, mice were deeply anesthetized and sacrificed by perfusion. First, animals were perfused with about 40 mL of PBS 1x/1 kU sodium heparin, and then with about 40 mL di PFA 4% in PBS 1x. After the perfusion the brain was collected and post-fixed overnight in PFA 4% in PBS 1x at 4°C. The day after the solution with PFA was substituted with a 30% sucrose solution at 4°C to allow cryoconservation of tissues. The brains were conserved in sucrose solution for two days, until they could reach the bottom of the vial. Finally brains were frozen in dry ice and conserved at -80°C until tissue preparation.

Brains were dissected with a microtome, this instrument allows to dissect frozen tissue, but also to conserve slices collected in free-floating. This choice was made because the friability of the ischemic tissue didn't allow vibratome cutting, and also because the free-floating permit to obtain a better staining. Sections were collected between coordinates AP = 1.40 and AP = -0.45. For every four collected, five were discharge and then conserved at -20°C in Storage Solution (30% Sucrose, 30% ethylene glycol in PBS 0.1%).

3.2. IMMUNOISTOCHEMISTRY

Slices for immunofluorances staining were choose and then colored in co-staining at 1:200. Different stainings were performed to assess neuroregeneration, neuroinflammation and vasogenesis.

- PCNA + NeuN
- DCX + PCNA
- Glut-1 + PCNA
- Iba1 + GFAP

(PCNA Proliferating cell nuclear antigen, Ms Mab IgG2a, Cell Signalling Technology, Beverly, MA, USA)

(NeuN Neuronal Nuclei, Ms Mab IgG1, Millipore, Billerica, MA, USA)

(DCX Doublecourtin (N-19), Goat, Santa Cruz, CA, USA)

(Anti-Glut1 Glucose Transporter-1, Calbiochem, USA)

(Anti-Iba1, Rabbit, Wako Chemicals, Richmond, VA, USA)

(GFAP, Monoclonal Anti Glial Fibrillary Acidic Protein, Sigma, St Louis (MO), USA)

Secondary antibodies were used at concentration of 1:100 because conserved in 50% glycerol with the following combination, respectively:

- FITC, Goat anti-Mouse IgG, Fcy Subclass 2a specific + Cy3, Goat anti-Mouse IgG, Fcy Subclass 1 specific (Jackson Immuno, USA)
- FITC, Donkey anti-Goat, IgG + AlexaFluor 647, Donkey anti-Mouse, IgG (Jackson Immuno, USA)
- FITC, Donkey anti-Rabbit, IgG + AlexaFluor 647, Donkey anti-Mouse, IgG (Jackson Immuno, USA)

- FITC, Donkey anti-Mouse, IgG + AlexaFluor 647, Donkey anti-Rabbit, IgG (Jackson Immuno, USA)

METHODS

Section were incubated in Antigen Retrieval solution (10mM Sodium Citrate pH 6.0, 10mM Tris-HCl pH 6.0) for 2 minutes at >85°C and then washed two times in PBS 1x. Later, slices were permeabilized for 30 minutes in 0.3% H_2O_2 and then washed again two times in PBS 1x. Slices were blocked for 1 hour on 5% normal donkey serum (NDS) and 0.1% PBSTx at RT. After other washes, slices were incubated overnight at 4°C with the primary antibody with 1% NDS and 0.1% PBSTx. The day after slices were washed two times in 0.1% PBSTx and once in PBS 1x and then incubated with the secondary antibody for 2 hours at room temperature. After three other washes slices were finally mounted onto gel coated slides and covered (FluorSave mounting medium, Calbiochem, USA). Once that slides were dried, they were examined under confocal microscope at 5x and 20x magnitude in the hippocampal area.

4. RESULTS

Neuroprotective effects by deletion of CD36 receptor were evaluated in a murine model of unilateral MCAo. Mice were divided in four experimental groups: one group wild type (WT), and one group knockout for CD36 that received MCAo (CD36$^{-/-}$); and two groups of sham animals that received the same treatment but without the occlusion by the intraluminal filament (sham WT and sham CD36$^{-/-}$).

Initially we monitored the recovery from the injury induced. In order to do this, we recorded weight and temperature variations until the end of the experiment. As shown in figure 41 temperature is significantly different between WT group and sham WT in different days during the experiment underling the difficulty to recover for lesioned mice.

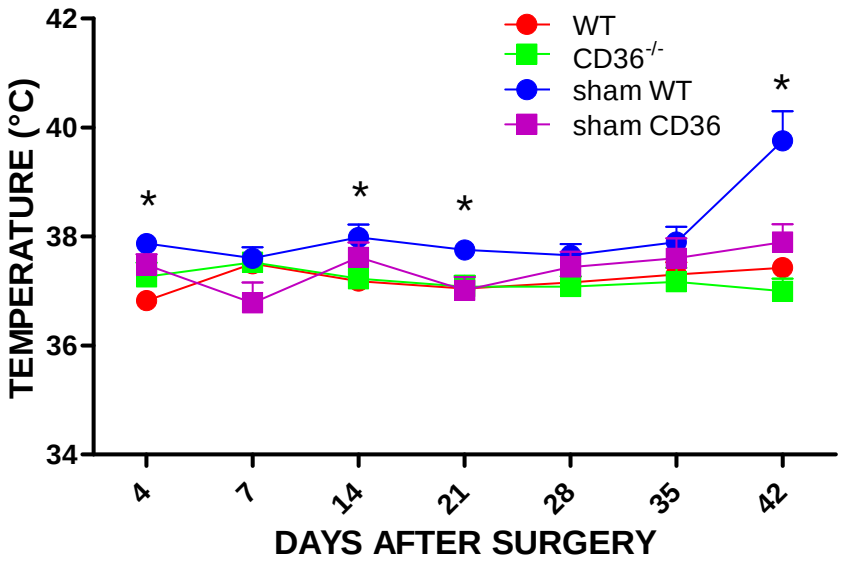


Figure 41. Temperature variations during 6 weeks. Temperature was taken once a week after the surgery for six week. Values are expressed as mean±SEM (n=10) of the temperature (°C). (**4 day:** p<0.01, WT vs sham WT; **14 day:** p<0.01, WT vs sham WT; **21 day:** p<0.05, WT vs sham WT; **42 day:** p<0.0001, WT vs sham WT; p<0.05, CD36$^{-/-}$ vs sham CD36$^{-/-}$; ANOVA, post-hoc test Bonferroni).

More interestingly it is not possible to see the same difference in CD36 knockout mice; this condition shows a faster recovery in CD36$^{-/-}$ mice than in WT group. On the other hand, the variation in the body weight of sham groups (considered as the percentage of the difference in the body weight between the day of the test and the day before the surgery) is significantly different with respect to lesioned mice (Fig. 42).

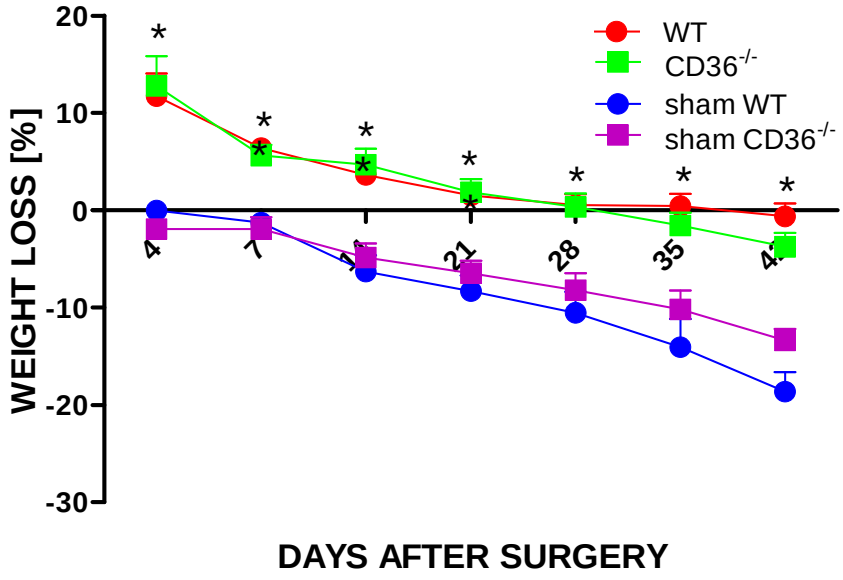


Figure 42. Weight loss percentage variation during 6 weeks. Mice were weighted once a week after the surgery for six week. Values are expressed as mean±SEM (n=10) of the weight (g) compared to the weight of the same animals the day before the surgery. (**4 day:** p<0.05, WT vs sham WT, **7 day:** p<0,0001, WT vs sham WT; p<0,001, CD36$^{-/-}$ vs sham CD36$^{-/-}$, **14 day:** p<0,001, WT vs sham WT; p<0,001, CD36$^{-/-}$ vs sham CD36$^{-/-}$, **21 day:** p<0,001, WT vs sham WT; p<0,01, CD36$^{-/-}$ vs sham CD36$^{-/-}$, **28 day:** p<0,001, WT vs sham WT; p<0,05, CD36$^{-/-}$ vs sham CD36$^{-/-}$, **35 day:** p<0,001, WT vs sham WT; p<0,001, CD36$^{-/-}$ vs sham CD36$^{-/-}$ **42 day:** p<0,0001, WT vs sham WT, p<0,01; CD36$^{-/-}$ vs sham CD36$^{-/-}$; ANOVA, post-hoc test Bonferroni).

Subsequently, we investigated the extension of the lesion using the modified Bederson test and the Corner test. The first test permitted to define the neurological assessment using a score presentation enclosed by 0 and 4. As we expected, figure 43 shows an important difference within sham groups and lesioned mice during the time course of our experiment, this result confirmed the validity of our MCAo model.

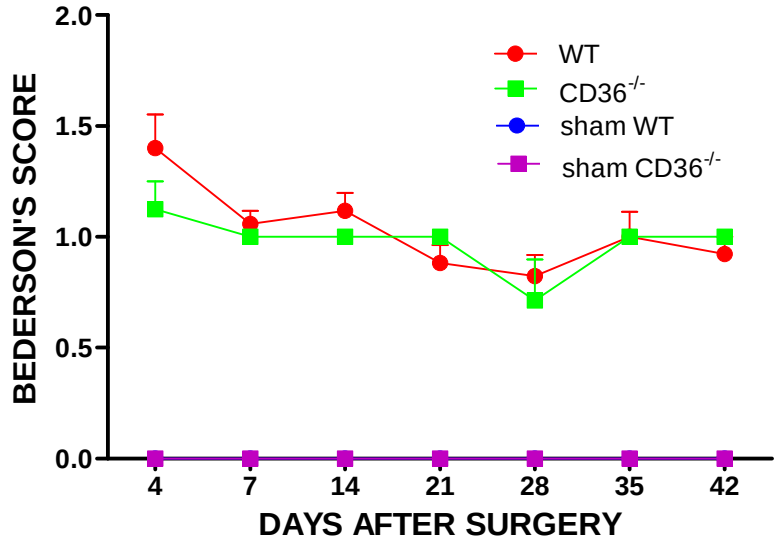


Figure 43. Variations in Bederson test evaluation during 6 weeks. Mice were tested and scored with a value between 0 and 4 to exprime motorial functions' compromisation. Data are expressed as mean ± SEM (n=10) of the value attribued to each animal.

The Corner test permitted to evaluate sensory motor integration and the extension of the lesion (Fig. 44).

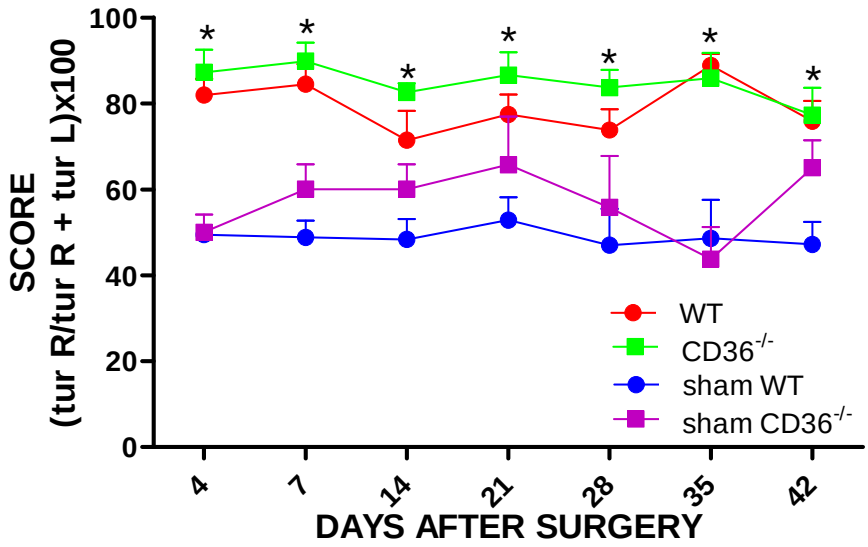


Figure 44. Corner test evaluation during 6 weeks. Mice were tested and scored once a week. Values are expressed as mean±SEM (n=10) of the percentual number of rotation in both side in 10 minutes. (**4 day:** p<0.0001, WT vs sham WT; p<0.0001, CD36$^{-/-}$ vs sham CD36$^{-/-}$; **7 day:** p<0.001, WT vs sham WT; p<0.05, CD36$^{-/-}$ vs sham CD36$^{-/-}$; **14 day:** p<0.05, WT vs sham WT; p<0.01, CD36$^{-/-}$ vs sham CD36$^{-/-}$; **21 day:** p<0.05, WT vs sham WT; **28 day:** p<0.05, WT vs sham WT; **35 day:** p<0.0001, WT vs sham WT; p<0.001, CD36$^{-/-}$ vs sham CD36$^{-/-}$; **42 day:** p<0.01, WT vs sham WT; ANOVA, post-hoc test Bonferroni).

Lesioned mice are not able to percept the board with the right vibrissae and consequently those rotations may be predominantly to the lesioned hemisphere (right). We demonstrated a consistent difference between lesioned mice and sham groups throughout six weeks, especially for the WT group compared to the equivalent sham group.

In addiction we evaluated muscular strength with the Hanging wire test to define differences in our experimental groups. Our results highlight a significant decrease of strength in WT group as compared to CD36$^{-/-}$ ($p<0.001$) 7 days after the surgery. On the other hand, we evidenced many difference between sham groups and lesioned mice but not between WT and CD36$^{-/-}$, especially from day 28 after the surgery. Figure 45 shows clearly, during the entire time course, an evident decrease in the strength of WT mice.

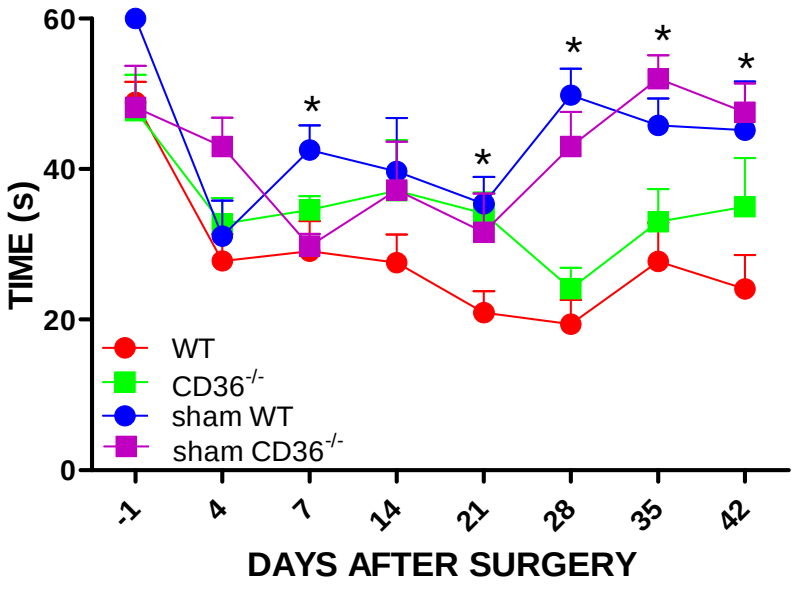


Figure 45. Muscular strength evaluation by hanging wire test during 6 weeks. Mice were tested once a week. Values are expressed as mean value±SEM (n=10) of the time (s) that the animal spent on the wire. (**7 day:** $p<0.001$, WT vs CD36$^{-/-}$; t test, Welch's Correction; **21 day:** $p<0.05$, WT vs sham WT; **28 day:** $p<0.001$, WT vs sham WT; $p<0.05$, CD36$^{-/-}$ vs sham CD36$^{-/-}$; **35 day:** $p<0.05$, WT vs CD36$^{-/-}$; **42 day:** $p<0.01$, WT vs sham WT; ANOVA, post-hoc test Bonferroni).

Furthermore, we considered the coordination and balance with the Pole test. This test permits to evaluate the first skill through the time that animals need to rotate on himself to result facing down on the top of the pole. The time required was related with the time taken from the same animal the day before the surgery. In this way was possible to establish that WT lesioned mice required much more time for the rotation than sham animals (4 days: $p<0.001$; 14 days: $p<0.0001$; 42 days: $p<0.05$, Fig. 46a). Interestingly, sham groups maintained the same time for the rotation, while we observed a progressive improvement in the task for $CD36^{-/-}$ lesioned mice. Balance involvement was studied through the time spent to descend the pole. As for the rotation time, we related the duration of the task with the performance of the same animal the day before the surgery. The figure 46b shows a significant difference between MCAo mice and sham groups, but nothing relevant among the lesioned groups.

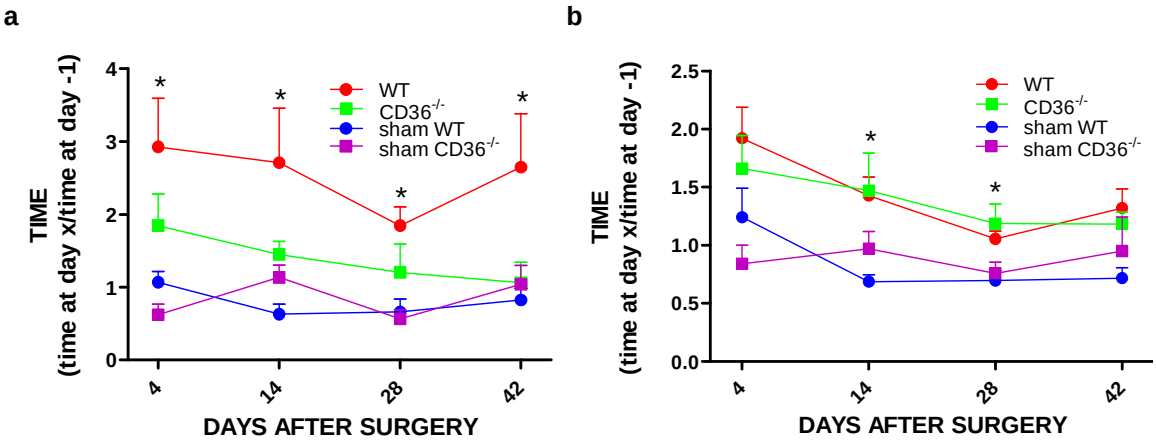


Figure 46. Pole test evaluation of coordination (time to rotate, a) and balance (time to descend, b) during 6 weeks. Mice were tested every two weeks. Values are expressed as the mean value±SEM (n=10) of the relative time required to turn (a) or descend (b) the pole and the time spent for the same task the day before the surgery. (**a: 4 days:** $p<0.001$, WT vs sham WT; $p<0.05$, $CD36^{-/-}$ vs sham $CD36^{-/-}$; **14 days:** $p<0.001$, WT vs sham WT; $p<0.0001$, WT vs $CD36^{-/-}$; **28 days:** $p<0.05$, $CD36^{-/-}$ vs sham $CD36^{-/-}$; **42 days:** $p<0.05$, WT vs sham WT. **b: 14 days:** $p<0.001$, WT vs sham WT; **28 days:** $p<0.01$, WT vs sham WT; t test, Welch's correction).

Finally, in view to evaluate the sensitivity to perceive an unknown object and the ability to control faint movements, we performed Sticky tape test.

This test permits to study many different aspects of the motor coordination and their related damages. We used the time to enter in contact for the first time with the tape as a proof of the sensitivity in perception. As shown in figure 47, the time required to contact the contralateral side (controlled by the lesioned hemisphere) related to the ipsilateral side (controlled by the healthy hemisphere) is absolutely equivalent for all experimental groups before the surgery, but it is significantly higher in lesioned mice than in sham mice after the lesion induced. We observed a significant difference between WT mice and the respective sham group, but not between the other two experimental groups (7 days: $p<0.01$; 21 days: $p<0.01$; 35 days: $p<0.01$).

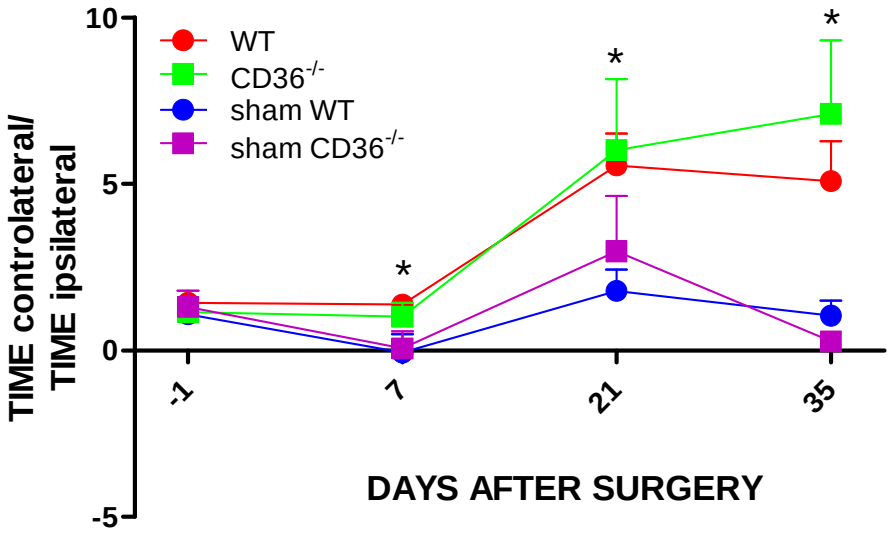


Figure 47. Time to contact in the sticky tape test during 6 weeks. Mice were tested every two weeks. Values are expressed as mean value±SEM (n=10) of the relative time that animals need to contact the contralateral paw and the time for the ipsilateral side. (**7 days:** $p<0.01$, WT vs sham WT; **21 days:** $p<0.01$, WT vs sham WT; **35 days:** $p<0.01$, Wt vs sham WT; $p<0.05$, $CD36^{-/-}$ vs sham $CD36^{-/-}$; t test, Welch's correction).

In order to evaluate the ability to control faint movements, we estimated the time spent by animals to remove an unknown object from the moment of the first perception of it. We related the time to remove the contralateral side to the ipsilateral side, as shown in figure 48, before the MCAo, all mice showed a similar relation, comparable to one. By contrast, from the seventh day after the lesion, although CD36 knockout mice resulted to be impaired

compared to the respective sham group (7 days: p<0.01, CD36$^{-/-}$ vs sham CD36$^{-/-}$; 21 days: p<0.05, CD36$^{-/-}$ vs sham CD36$^{-/-}$; 35 days: p<0.01, CD36$^{-/-}$ vs sham CD36$^{-/-}$), WT animals were much more compromised as compared to the respective sham animals (7 days: p<0.01, WT vs sham WT; 21 days: p<0.01, WT vs sham WT; 35 days: p<0.01, WT vs sham WT). More interestingly, throughout the experiment we observed a significant difference between WT lesioned mice and CD36$^{-/-}$ lesioned animals (7 days: p<0.05, WT vs CD36$^{-/-}$; 21 days: p<0.01, WT vs CD36$^{-/-}$; 35 days: p<0.01, WT vs CD36$^{-/-}$).

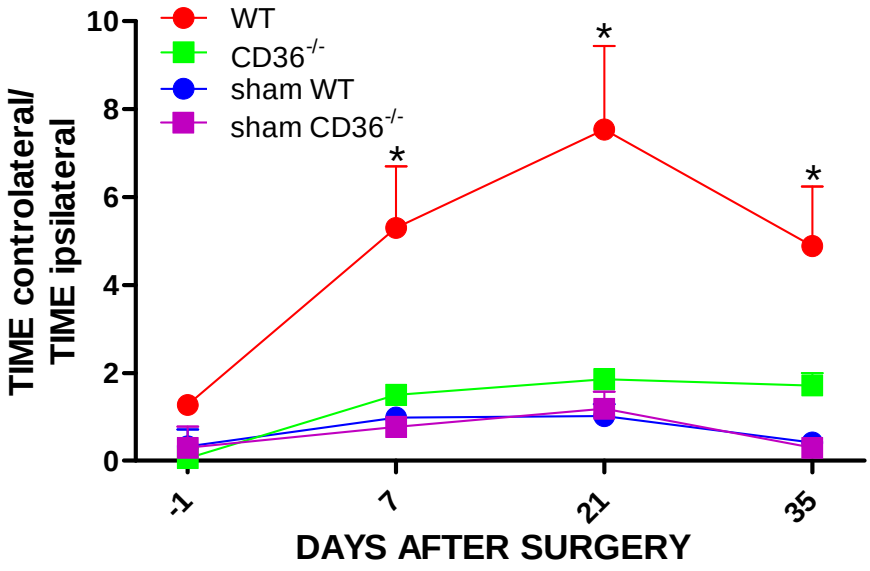


Figure 48. Time to remove in sticky tape test during 6 weeks. Mice were tested every two weeks. Values are expressed as mean value±SEM (n=10) of the relative time that animals spent to remove the contralateral and the ipsilateral side. (**7 days:** p<0.01, WT vs sham WT; p<0.01, CD36$^{-/-}$ vs sham CD36$^{-/-}$; p<0.05, WT vs CD36$^{-/-}$; **21 days:** p<0.01, WT vs sham WT; p<0.05, CD36$^{-/-}$ vs sham CD36$^{-/-}$; p<0.01, WT vs CD36$^{-/-}$; **35 days:** p<0.01, WT vs sham WT; p<0.01, CD36$^{-/-}$ vs sham CD36$^{-/-}$; p<0.05, WT vs CD36$^{-/-}$; t test, Welch's correction).

The last parameter that we investigated through the sticky tape test was the latency to respond, as the recorded time between the time for the removal and the time of the first contact always by relating the contralateral and ipsilateral sides. In this case, we observed that WT animals needed a longer time than CD36$^{-/-}$ mice to assess the task (7days: p<0.05, WT vs CD36$^{-/-}$; 21 days: p<0.01, WT vs CD36$^{-/-}$; Fig. 49), showing a better outcome

of knockout mice. This parameter is extremely important because it is strictly related to the ability to the control of the faint movements.

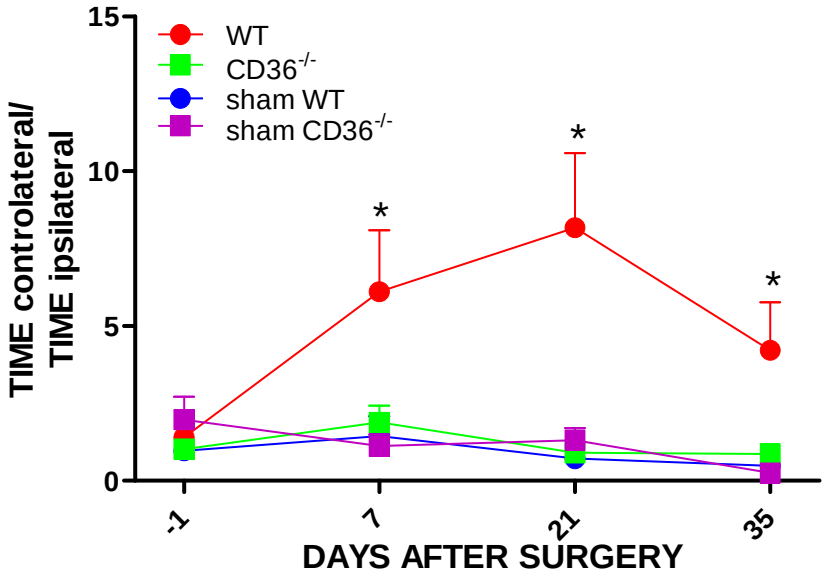


Figure 49. Latency to remove the tape from the first time of contact in the sticky tape test during 6 weeks. Mice were tested every two weeks. Values are expressed as mean value±SEM (n=10) of the relative time spent to remove the tape after the contact in the contralateral and ipsilateral side. (**7 days:** $p<0.05$, WT vs sham WT; $p<0.05$, WT vs CD36$^{-/-}$; **21 days:** $p<0.01$, WT vs sham WT; $p<0.01$, WT vs CD36$^{-/-}$; **35 days:** $p<0.05$, WT vs sham WT; $p<0.05$, CD36$^{-/-}$ vs sham CD36$^{-/-}$; t test, Welch's correction).

Starting from the third week after the MCAo induction, we assessed cognitive behavioral tests to elucidate the possible impairment of declarative and non-declarative memory. For this purpose, we performed the Novel object recognition test. The test permits to evaluate the time spent by animals in the exploration of a new object. Our results did not show a better outcome in knockout animals as compared to WT. On the other hand, the time spent in the exploration of the unknown object, is higher in the sham groups than in the lesioned mice (Fig. 50).

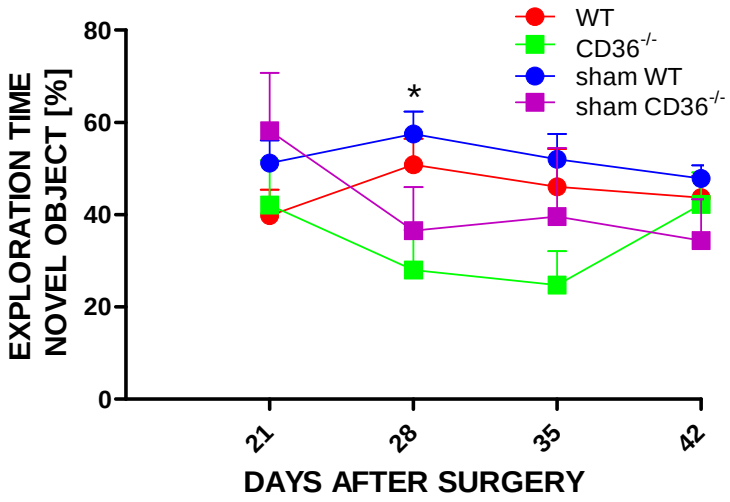


Figure 50. Novel Object Recognition Test during 6 weeks. Mice were tested every week from the third week after the surgery. Values are expressed as the mean value±SEM (n=10) of the percentual time (%) spent in the exploration of the novel object. (**28 days:** $p<0.05$, WT vs CD36$^{-/-}$; ANOVA; post-hoc test Bonferroni).

As in previous cognitive test, also the Y-Maze test could be used to consider the involvement of non-declarative memory. The test permits to evaluate the time spent by mice in the exploration of a new environment. Our results did not show any difference among the four experimental groups (Fig. 51).

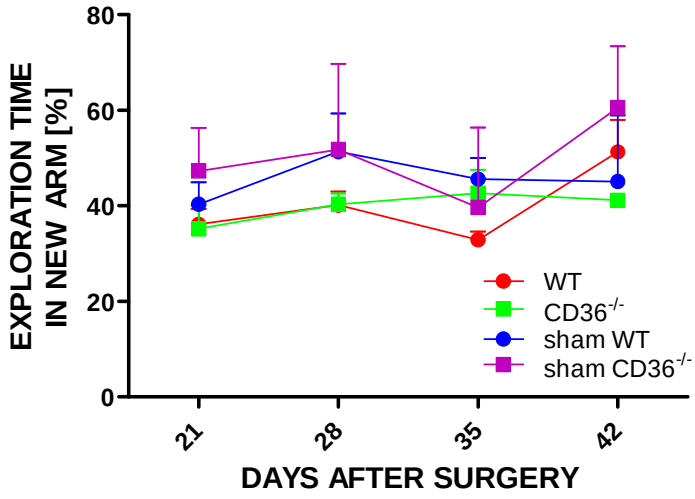


Figure 51. Y-Maze test during 6 weeks. Mice were tested once a week from the third week after the surgery. Values are expressed as mean value±SEM (n=10) of the percentual time (%) spent in the exploration of the new arm.

Finally, after the sacrifice, tissues were collected and we proceeded with immunohistofluorescence analysis of the sections collected at microtome. Figure 52 shows an example of hippocampal area lesioned (right) and contralateral (left) in WT, CD36$^{-/-}$ and shams groups stained with Neun (red) and PCNA (green). Images show neurons and neurons in division at 42 days after the surgery, presenting how the hemisphere lesioned of knockout mice has some neurons in division.

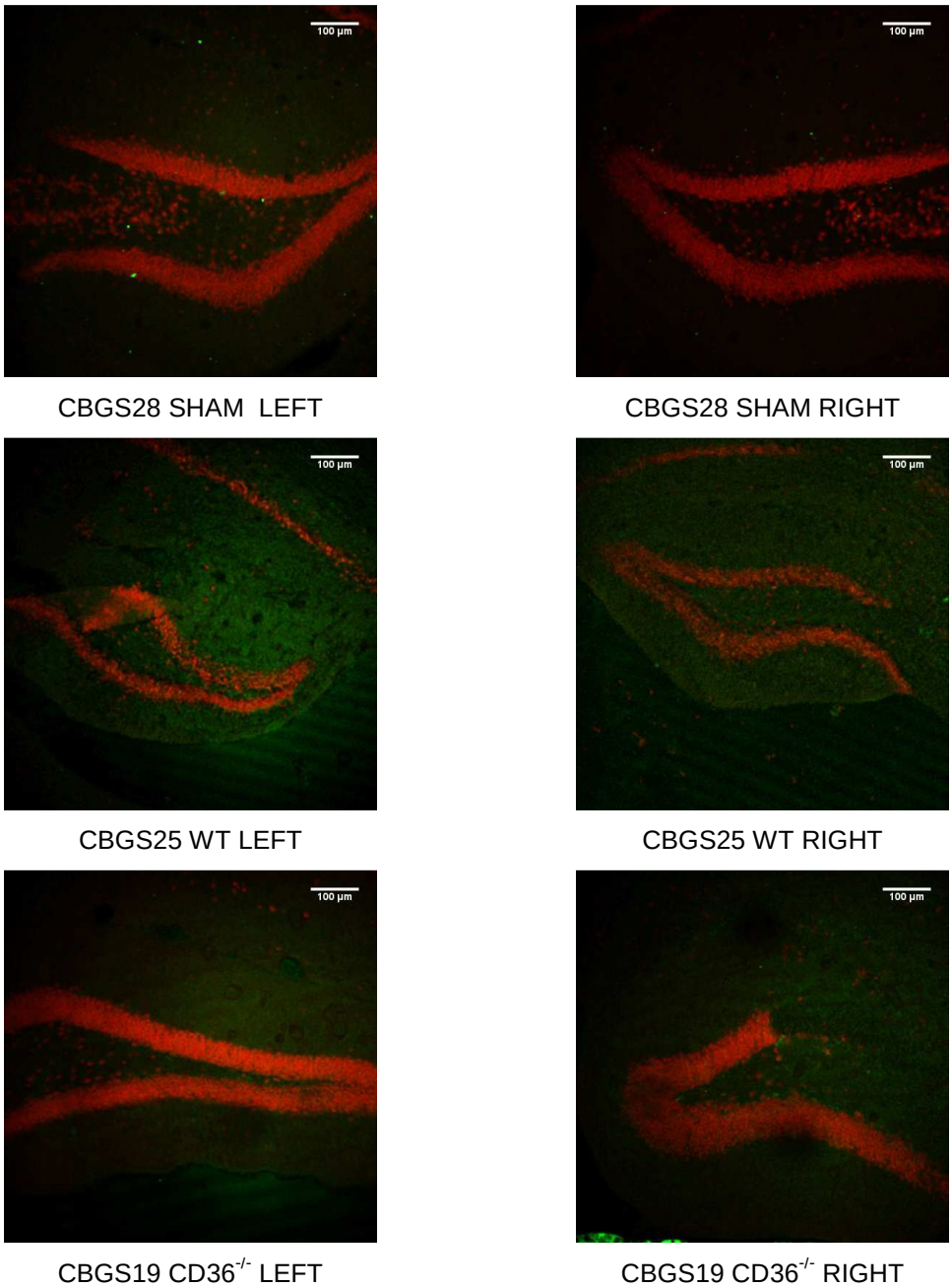


Figure 52. PCNA+NeuN. Representative images of hippocampal area. Magnitude 20x, scale bar 100 µm. PCNA green by FITC anti-mouse IgG2a, NeuN red by Cy3.

In order to localize angiogenic process and relate it to the neurodegenerative process, we stained together Glut1 and PCNA in WT lesioned and sham mice (Fig. 53). Interestingly, we noticed that the process was prevalent in MCAo mice than in sham animals. Moreover, the damage induced by the occlusion was expanded to the hippocampal area where is possible to notice the angiogenic process.

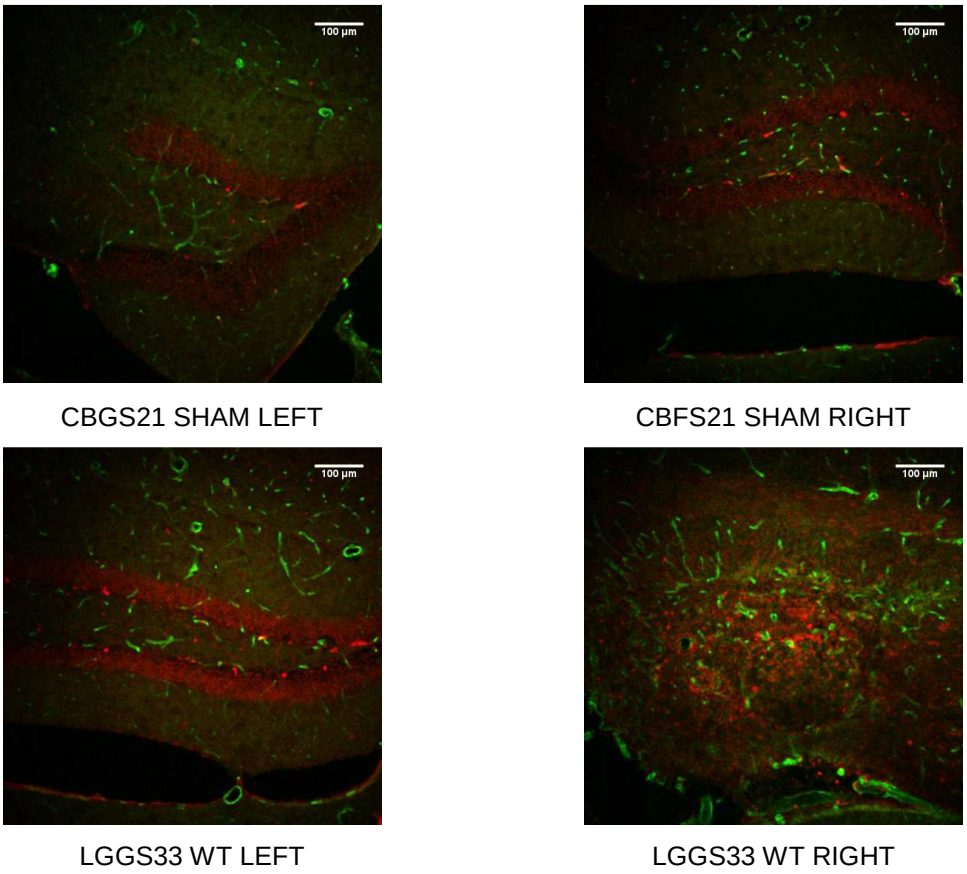
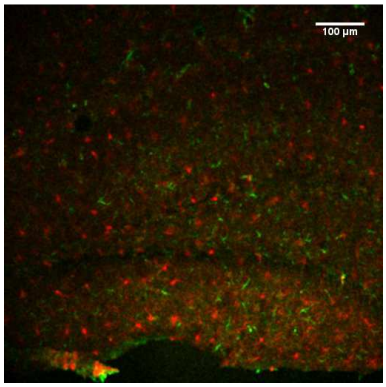
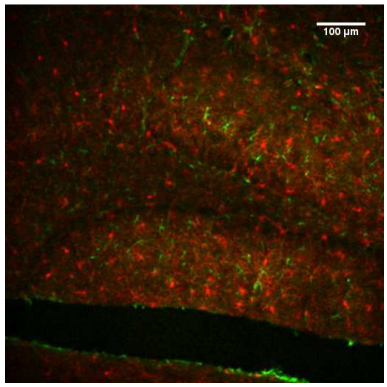


Figure 53. PCNA+Glut1. Representative images of hippocampal area. Magnitude 20x, scale bar 100 µm. Glut1 green by FITC anti-rabbit, PCNA red by AlexaFluor 647 anti-mouse.

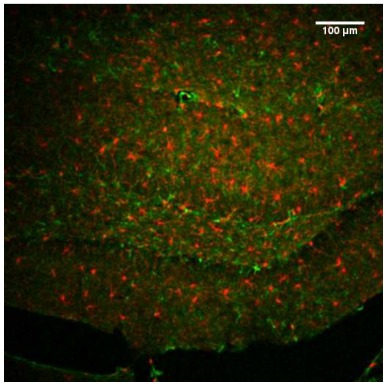
We also investigated inflammatory response in the long term development after the injury induced; at this purpose we stained Iba1 with GFAP to localize microglia and astrocytes (Fig. 54). Pictures illustrate that as in sham group responses are equivalent in any hemispheres, on the other hand WT show an higher microglia expression in the lesioned side, while $CD36^{-/-}$ illustrates more astrocytes activation.



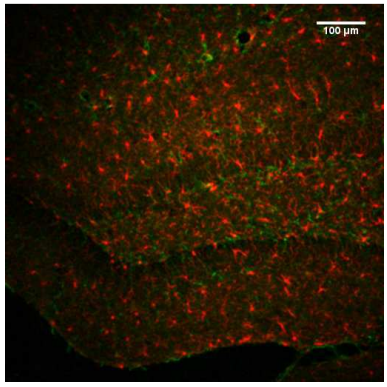
CBGS28 SHAM LEFT



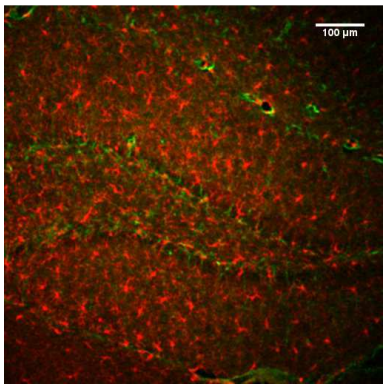
CBGS28 SHAM RIGHT



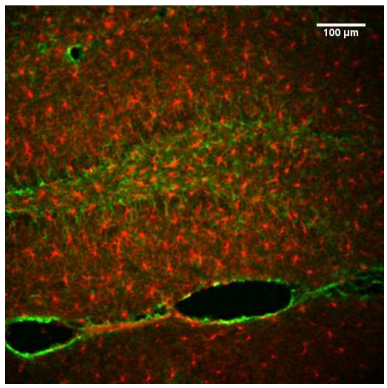
LGGS34 WT LEFT



LGGS34 WT RIGHT



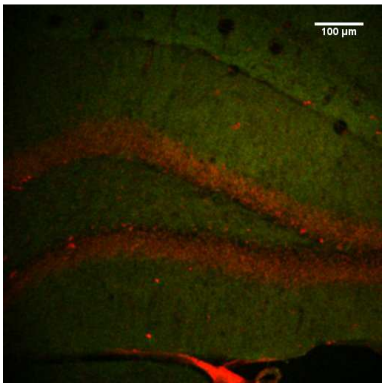
CBGS22 CD36$^{-/-}$ LEFT



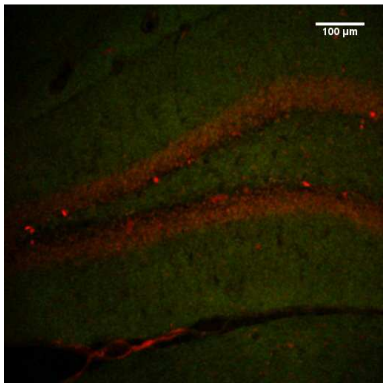
CBGS22 CD36$^{-/-}$ RIGHT

Figure 54. GFAP+Iba1. Representative images of hippocampal area. Magnitude 20x, scale bar 100 µm. GFAP green by FITC anti-mouse, Iba1 red by AlexaFluor 647 anti-rabbit.

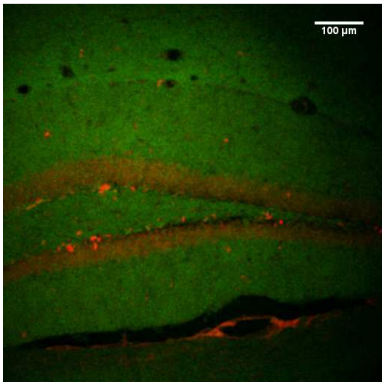
Finally, we investigated the relation between neuronal migration and neurogenesis process in hippocampal area. In this order we stained DCX and PCNA, as shown in figure 55 CD36$^{-/-}$ mice present a higher neurons in division than in WT animals.



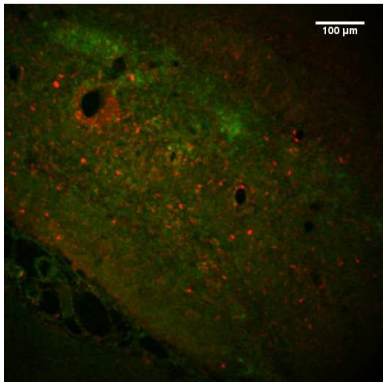
CBGS28 SHAM LEFT



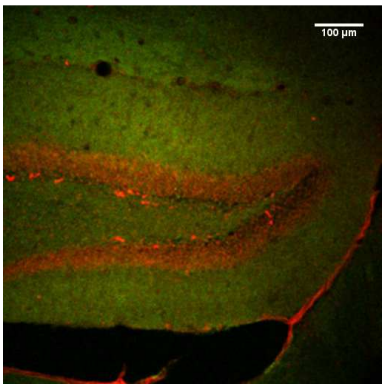
CBGS28 SHAM RIGHT



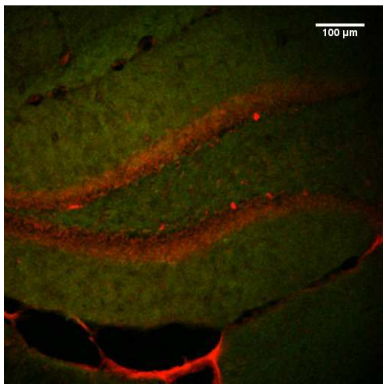
LGGS32 WT LEFT



LGGS32 WT RIGHT



CBGS22 CD36$^{-/-}$ LEFT



CBGS22 CD36$^{-/-}$ RIGHT

Figure 55. DCX+PCNA. Representative images of hippocampal area. Magnitude 20x, scale bar 100 µm. DCX green by FITC anti-goat, PCNA red by AlexaFluor 647 anti-mouse

5. DISCUSSION

Cerebral stroke is one of the major causes of death in industrialized countries (124). Vascular inflammation and oxidative stress are the main pathways through which stroke risk factors enforce their damaging effects on blood vessels. In particular, we focused our attention on the role of inflammation and how it may be modulated to obtain a better motor and cognitive outcome.

Inflammation is a double faced mechanism, on the one hand determines an increased cerebral blood flow to the affected area and the removal of damaged tissue by phagocytic cells; on the other hand, secretory factors such as cytokines and chemokines may reach toxic levels, besides the incremented ROS production and the consequent destruction of the BBB (196).

CD36 receptor is a class B scavenger receptor responsible for the regulation of physiological and pathological functions. The activation of CD36 is involved in several risk factors for stroke disease. In particular its activation determines an inflammatory response through the involvement of NFκB (209).

Cerebral ischemia determines an important impact on motor ability and cognitive functions (148, 149). In this context, it is clear that this type of acute degeneration may be easily compared with the dysfunctions caused by other neurodegenerative chronic diseases. In fact several motor behavioral test that we used in this experiment, were previously used for PD, as the cognitive tests were used for AD. Keeping in mind that many processes underling the evolution of the pathology are strictly related with chronic neurodegenerative diseases, the gap between these two types of CNS disorders has not to be considered so wide.

For this reason, we decided to investigate the potential contribute of CD36 receptor, as fundamental key point in the modulation of the outcome after stroke. Several in vivo studies have shown motor deficit in mice after MCAo, especially in the first days after the injury (213). We decided to perform a long term study (six weeks) to assess motor and cognitive task with the intent to underlie how the suppression of CD36 receptor may have a crucial role in the extension of the lesioned area after cerebral ischemia. We used a transgenic mouse model CD36 knockout, which has been surgically induced MCAo by the insertion of an intraluminal filament. From the fourth day after the lesion for six weeks, we performed behavioral motor skills to investigate coordination, balance, strength and sensitivity. Moreover from the third week after the surgery we also performed cognitive behavioral tests to evaluate the involvement of declarative and non-declarative memory.

In first instance, our results showed that CD36 knockout mice demonstrated a better recovery from the surgery than WT, this confirmed what previously assessed by Cho et al, indicating that the suppression of inflammation could be a key target for the modulation of the pathology (208).

For what that concern the extension of the lesion we used modified Bederson test and the corner test. In our experiment, both MCAo groups presented, as we expected, significant variation compared to sham groups, even if the difference observed in WT mice is slightly more evident.

After that, we evaluated the muscular strength by the hanging wire test, and we found that WT animals, after the lesion induced, had a significant loss of strength, and the different is much more important in the first evaluation after the damage, as shown by Wang et al in the early stages after the stroke induced (214).

Furthermore, we considered the balance and the coordination of animals by pole test. Interestingly, we observed an important decrease in coordination ability in WT lesion mice than in $CD36^{-/-}$ damaged animals but the variation in balance capacity were observable only through WT groups.

Our results are supported by Linden et al that showed a consistent impairment in balance and coordination in MCAo mice compared with sham animals in a short term experiment than ours (215). The performance of $CD36^{(-/-)}$ may be indicative for a better motor outcome in transgenic mice after cerebral stroke induction.

Finally, the sticky tape test has permitted to establish the ability to control faint movements and the sensitivity to perceive an unknown object. Our results showed through the time to contact the first time the tape that both lesioned groups presented a longer time than sham groups, especially WT animals during the entire time course. The ability to control faint movements was particularly compromised in WT animals than in transgenic mice, but we would like to focalize on the significant difference that we observed between lesioned groups. The last consideration on the sticky tape test is about the latency time between the first contact with the tape and its removal, this data is useful to confirm what we earlier observed with the time to remove the tape. Thus, the CD36 suppression results in less motor impairment in our murine model of cerebral ischemia.

Unfortunately, the cognitive observations didn't show what we expected. Either novel object recognition test or Y-maze test showed any consistent differences among our four experimental groups. Our results are confirmed by Bouët et al, they previously observed that ischemia did not alter spatial learning abilities, as we found in Y-maze test (180). Furthermore, Blasi et al in a subcortical white matter stroke experiment did not observe any differences in Y-maze test but, by contrast, they showed a significant impairment in novel object recognition test (216). In this contest we have to consider some experimental aspects. In our experimental time course, we decided to perform cognitive evaluation from the third week after surgery every week. This decision may have determined that, if animals didn't have an extended lesion, probably they could remember from one trial to the other what they already performed previously. In this case it may be not possible to show a consistent difference between lesioned mice and

sham groups because probably not completely lesioned animals could remember the object (or the environment) and then the result of the test would be compromised. The last consideration is about the animals' age, indeed, a human experiment on cognitive deficits after a stroke event, showed that only the 20.4-34.8% of the young patients compared to 77% of elderly stroke survivors presented cognitive features. Thus, 8 weeks of age for mice may be not enough to show a significant cognitive impairment. This may due partially to the fact that younger stroke survivors have a better collateral blood supply (217).

After the behavioral analysis mice were sacrificed and we proceeded with immunofluorescent investigation. Pictures presented are representative examples of our work. They show that the tissue is better conserved in CD36 knockout mice than in WT animals. WT animals displayed more lesions and, as we expected, in the lesioned hemisphere, the angiogenetic process is more extensive in the damaged area. The better conservation of tissue in $CD36^{(-/-)}$ mice may be explained by the study of Cho et al which showed the anti-angiogenic role of CD36, that support also the better behavior outcome observed in knockout mice (208). The neurogenesis process is not only more representative in $CD36^{-/-}$ mice, but also after the lesion in both groups. The inflammation response by microglia and astrocytes showed a higher activation after the MCAo, and the activation is still present in $CD36^{-/-}$ animals. Probably the activation of a mild inflammatory response would be important to explain the raised neurogenic process; indeed Chen et al explained that the activation of microglia following a brain injury may actively enhance neurogenesis by producing insulin-like growth factor-1 (218). Our considerations about the importance of CD36 receptor in the outcome after stroke are confirmed by Kim et al, who showed in an acute ischemia induced study, the protective effects of targeting the receptor of our interest (219). Besides these considerations, our study needs further investigations.

In conclusion, we can affirm that the knockout of CD36 receptor could have a protective role in neuroinflammation after a stroke event as shown by motor impairments considerations. From the moment that many mechanisms that underlie ROS production and spreading brain inflammation are common in both acute and chronic neurodegenerative diseases, it will be interesting to investigate the role of CD36 also in other neurodegenerative pathologies and how it is possible to modulate the receptor in order to obtain a slower progression of the brain lesion.

Conclusions

Different neurodegenerative diseases have shown to share similar dysfunctional features. Although if the epidemiology of the most of them is not certain yet, especially for neurodegenerative chronic diseases, oxidative stress, inflammation, excitotoxicity and neuronal loss are extremely involved in the evolution and progression of the pathology, both in chronic and acute conditions. The brain is the organ more exposed to oxidative stress because of the high rate of oxygen consumption related to the detoxification mechanism. For this reason, any variation in this balance could play a dramatic role in the evolution of a brain damage (42). Inflammatory response and oxidative stress are closely related to each other by a double chain; increasing oxidative condition raises the inflammatory response that, in turn, causes oxidative stress. The complexity of the brain structure and functions still not permit to describe clearly how these pathologies could evolve, but it is possible to observe a superposition among them. For example many motor tests, already used in the study of PD, have shown a good outcome in the study of motor impairment after an ischemic cerebral event. This is possible because the brain structures involved in these two diseases are comparable. On the other hand, many patients present an AD condition after a history of cerebral ischemia due to an overexpression of β-amyloid plaques (12). All these considerations allow us to affirm that there is a tight junction between chronic and acute neurodegenerative diseases, although if the causes and the epidemiology are often distant and completely different.

In this context the concept of neuroprotection, as an intervention to slow down or arrest the evolution of pathology, may have a powerful role. Any intervention able to interfere with the inflammatory response and oxidative stress could play a crucial role in the progressive impairment of the quality life of patients. Neuroprotection could work in synergy with the

endogenous systems, quenching ROS formation or restoring the antioxidant GSH system and its related enzymes, as GR and GST; and not less important slowing down the apoptotic neuronal death. Thus, the present study we used a murine model of PD treated with a powerful antioxidant molecule, 6-MSITC. As already highlighted, inflammation is a fundamental characteristic in the evolution of neurodegenerative chronic and acute diseases; in fact various studies have shown how NSAIDs could exert neuroprotective effects in PD animal models (21, 38). For this reason, the modulation of this response is also a key point in the neuroprotection strategy. In the second study a transgenic mouse model knockout for CD36 receptor has been utilized to investigate the involvement of the inflammation in a long term study of MCAo. The results show that the suppression of this receptor determines a better outcome in mice after the induced damage; therefore, we think that a good neuroprotective therapy could be useful in a better outcome in ischemic patients.

In this work it has been shown how is possible to modulate a neuroprotective response by a powerful antioxidant molecule, 6-MSITC, in a preclinical murine model of PD and the modulation of inflammatory response by the suppression of CD36 receptor in a preclinical model of MCAo. Because of the common mechanisms involved in the spreading of neurodegenerative pathologies, chronic and acute neurodegenerative diseases are not so far each other as they seem to be. It will be interesting to investigate the neuroprotective activity of antioxidant molecules in cerebral ischemia, especially the possible action on the penumbra, the area where neurons appear damaged, but not irreversible compromised. On the other hand the CD36 receptor has shown interesting property in the control and evolution of the inflammatory response, and it would be interesting to investigate its role in chronic diseases.

In conclusion, results in this study allow underlying the connection among pathologies that involved CNS, and the importance of a

neuroprotective strategy able to restore neurons activity where current drugs therapies have shown palliative but not healing abilities.

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