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XXVII Ciclo**

Tesi di Dottorato

**IN VIVO AND IN VITRO CHARACTERIZATION OF
GASTROINTESTINAL DYSMOTILITY IN THE 6-OHDA RAT MODEL
OF PARKINSON'S DISEASE**

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*“What counts in the case of chronic disorders is the patient’s suffering,
not the nature of the disease”*

*“I have finally come to the conclusion that a good set of bowels is
worth more to a man than any quantity of brains”*

Enrico S. Corazziari
Chronic constipation
2013

Summary

The aetiology of Parkinson's disease (PD), an aging-related disease characterized by progressive degeneration of dopaminergic neurons of the substantia nigra is not fully clarified yet, and moreover, there is an ever-increasing understanding that this disease is more than a motor disorder. Research into the non motor symptoms of PD is the focus of intense investigations, and there is hope of developing treatments that not only arrest the progress of the disease but stop it in its tracks. Among the non motor autonomic disorders, complications in the gastrointestinal (GI) system are the most peculiar, which degrade the quality of life of the patient and may interfere, as in the case of changes in the normal gastrointestinal transit, with the proper absorption of drugs used in the treatment of the disease itself. Moreover they can appear in very early stage of the disease, or even before the onset of motor symptoms, until they have been mentioned as possible pre-clinical signs.

The purpose of the present thesis work was to characterize and study the effects of a central dopaminergic parkinsonian deficit, reproduced in Sprague Dawley rats bearing nigrostriatal 6-hydroxydopamine (6-OHDA) lesion, on the motor functions of the gastrointestinal tract. It has been adopted different *in vivo* methods to evaluate macroscopically the gastric motility and intestinal transit, and subsequently *in vitro* studies to verify the presence of any neuronal or receptor changes or any disruption caused by oxidative stress, and also to check the functionality of the pacemaker cells, Interstitial Cells of Cajal (ICC). In addition, it has been evaluated the evolution of gastrointestinal disorders following an oral subchronic four weeks treatment with L-DOPA/Benserazide, still considered a "gold standard" in the treatment of Parkinson's disease.

The *in vivo* results showed alterations of the gastrointestinal motility which develop in different time points following the whole course of the experiments. It has been demonstrated the presence of a delayed emptying of the stomach and a slowed intestinal transit after 8 weeks from 6-OHDA intracerebral injection, which can reflect the symptoms of gastroparesis and constipation detected in PD patients. The *in vitro* investigation provides evidence that 6-OHDA rats present a loss of responsiveness to exogenous contractile agents in gastric circular muscles and an impairment of pyloric

nitroergic control. To test the validity of the hypothesis regarding the role and involvement of ICC in gastroparesis, never investigated before in the experimental model adopted here, it has been performed an immunohistochemical study detecting a disruption of ICC plasticity consequently to the dopaminergic central deficit in the gastric regions of fundus, corpus and antrum.

As regards the oral subchronic treatment with L-DOPA/Benserazide, it shows a partial benefit after 8 weeks in terms of gastric and colonic dysmotility, bringing it to normal values. While it decreases the state of oxidative stress, detected by the index of lipoperoxidation MDA, in stomach and terminal colon.

In conclusion, this research has provided significant results and methodological knowledge. Comparing different types of investigations, it has been possible to identify the modalities that, in this specific experimental model endowed with translational validity, with higher reproducibility and limited variability, allow to monitor in vivo the gastrointestinal motor activity.

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Introduction

1. What is Parkinson's disease?

1.1. Brief history

What we know today as Parkinson's disease (PD) was first described by James Parkinson in his 1817 *Essay on the Shaking Palsy*, talking about "involuntary tremulous motion in parts not in action, with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellects being uninjured". However, it is probable that PD was present long before this landmark description. A disease known as *Kampavata*, consisting of shaking (kampa) and lack of muscular movement (vata), existed in ancient India as long as 4500 years ago (Ebadi et al., 2005). To treat this disease, indian people utilized seeds of a tropical plant called *Mucuna Pruriens*, natural source of therapeutic quantities of L-dopa (Ovallath S. et al., 2013).

It was not until more than 100 years after Parkinson's original description that the loss of dopamine-containing cells in the substantia nigra (SN), characteristic of PD, was recognized. Although neuropathological examination documented the distinctive presence of *Lewy Bodies (LB)* and degeneration of the SN as hallmarks of PD, no definitive clinical test or procedure to diagnose PD exists, and the diagnosis must be made on the basis of clinical features alone.

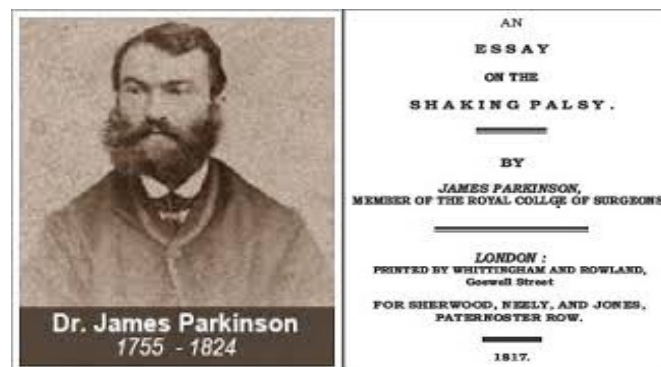


Fig.1. Dr James Parkinson (1755-1824) and his *Essay on the Shaking Palsy* (1817).

After Parkinson other neurologists have contributed to a better understanding of the disease as Trousseau, Gowers, Wilson, Erb and, more particularly, Jean-Martin Charcot, whose studies carried out between 1868 and 1881 were a landmark in the understanding of the disease. Charcot, in his essay "*Leçons sur les maladies du système nerveux*", described again this clinical condition, calling it Parkinson's disease. In 1919 Tretiakoff discovered that substantia nigra was the main brain structure to be affected, but the importance of this discovery was realized only in the mid-twentieth century, when the disease has been biochemically characterized.



Fig.2. The Parkinson Red Tulip

In 1980 J.W.S. Van der Wereld, a Dutch horticulturalist who suffered from Parkinson's disease, developed a red and white tulip, calling it "Dr. James Parkinson" to honor the man who first described the condition. In 2005, the red tulip was launched as a universal symbol of Parkinson's disease and it is used from the European Parkinson's Disease Association (EPDA) as its logo (Fig.2).

1.2. Epidemiology and general features of the disease

Parkinson's disease is the second neurodegenerative pathology in order of frequency after Alzheimer's disease (Tanner CM et al., 1996) with a prevalence of 1/400 for the general population and 1/200 for subjects over the age of 40 (Pinelli P et al., 1993). In 85% of cases the aetiology of Parkinsonian Syndromes is unknown; for these patients is referred to as Parkinson's disease (PD) or idiopathic parkinsonism, to differentiate it from Symptomatic Parkinsonisms, related to known aetiology.

Four cardinal signs constitute the core clinical complex of parkinsonism: tremor, akinesia or bradikinesia, rigidity, and loss of postural reflexes (Ebadi et al., 2005). These symptoms are due to a progressive neuronal degeneration in the substantia nigra, with subsequent decrease of cerebral contents of dopamine, main neurotransmitter synthesized at that level. The number of dopaminergic neurons decreases progressively during the disease course with twice the speed of about 1% per year, instead of 0.5% as in normal subjects (Scherman D et al., 1989). The symptoms begin to appear when the depletion of dopamine neurons exceeds the threshold of 70-80% at the level of striatal nerve terminals and 50-60% at the level of nuclei in the substantia nigra (Agid Y et al., 1991).

It is noteworthy that there is a general deterioration of neurons with age, including dopaminergic neurons: this event is physiological during aging, but in most of the population this loss does not reach the value of 70-80% necessary condition for the symptomatic onset of Parkinson's disease. The progress of the disease leads to a state of akinesia with stiffness for which the patient loses the self-sufficiency and death occurs frequently as a result of complications of immobility, including aspiration pneumonia associated with swallowing difficulties, and pulmonary embolism.

In addition to the abovementioned cardinal signs, a variety of additional motor features may develop in PD. Speech becomes both soft and poorly articulated. Dysphagia is often present and may lead to aspiration. Handwriting becomes micrographic. Posture becomes flexed and gait is characterized by small, shuffling steps on a narrow base (Fig.3).



Fig.3. Typical motor symptoms of the disease.

A number of non motor features also characterize PD, although they have received less attention. Autonomic abnormalities may include bowel dysfunction, urinary difficulties, sexual disturbances, cardiovascular changes, and thermoregulatory alterations. Behavioural changes, such as depression and anxiety, are frequently present in PD.

While no preventive or curative treatment for PD has been discovered to date, the evolution of treatment for PD has been characterized by a fascinating, and in many respects dramatic, progression to more effective symptomatic therapies. In tandem with these advances, therapeutic attention has also begun to focus on treatment that might actually alter or slow progression of the disease process itself (Ebadi et al., 2005).

2. Dopamine

2.1. Biosynthesis, release and degradation

Dopamine (DA) is a neurotransmitter which belongs to the family of catecholamines and is a precursor of norepinephrine. Its biosynthesis starts from tyrosine, an aromatic amino acid that is taken up by noradrenergic/dopaminergic neurons. The cytosolic enzyme tyrosine hydroxylase catalyzes the conversion of tyrosine to dihydroxyphenylalanine (DOPA). This first step of hydroxylation is the rate-limiting step of the whole process and is inhibited by the end product of the pathway, that is

norepinephrine. The next step consists of the conversion of DOPA to dopamine, catalyzed by L-aromatic amino acid decarboxylase. Dopamine may be an additional substrate for hydroxylation with the formation of norepinephrine (Fig. 4).

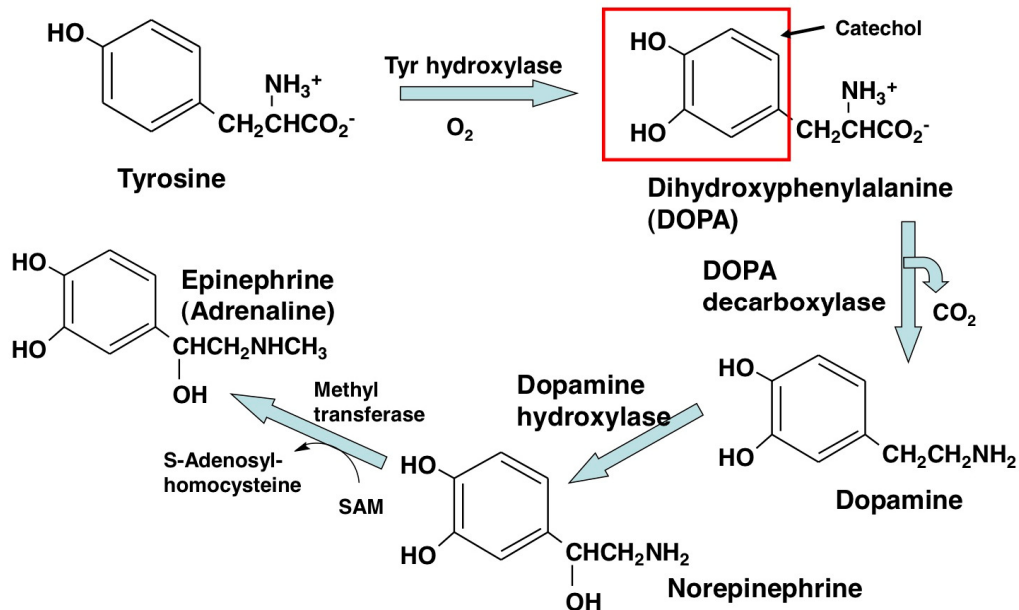


Fig.4. Biosynthesis of Dopamine.

Once dopamine has been synthesized, it is stored in vesicles from which it will be released into the synaptic space in response to a presynaptic action potential.

The action potential determines the depolarization of the membrane and opening of voltage-dependent calcium channels with the consequent entry of Ca^{2+} , which promotes the fusion of the vesicles with the membrane and the release of their contents. In the intersynaptic space, dopamine can bind to pre- or post-synaptic receptors or still be re-uptaken by specific transporters (dopamine transporter DAT) on nerve endings. Dopamine is then degraded by deamination, operated by the monoamine oxidase (MAO), and/or methoxylation, realized by catechol-O-methyltransferase (COMT), becoming homovanillic acid (HVA) (Fig. 5,6).

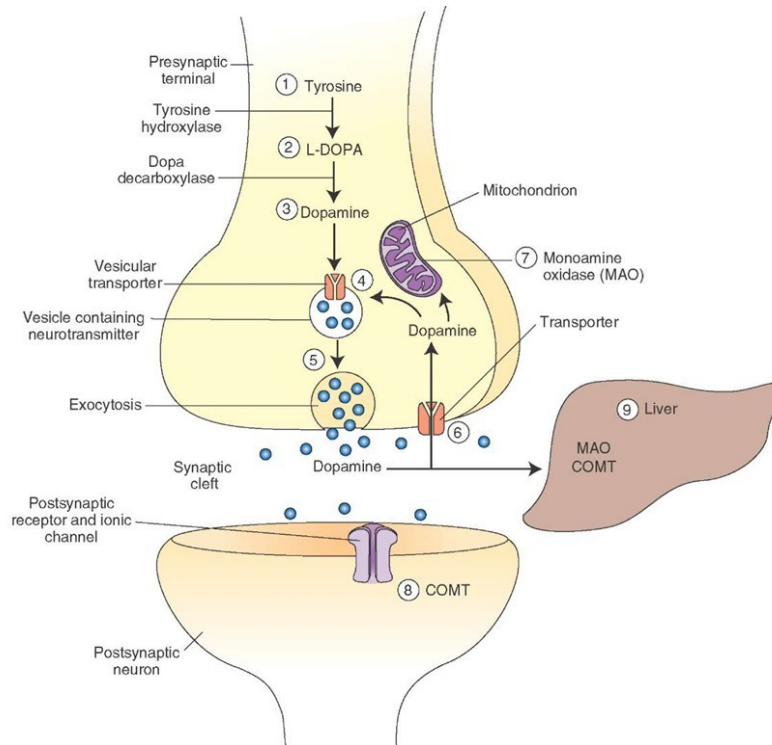


Fig.5. Biosynthesis, release in synaptic space and degradation of dopamine.

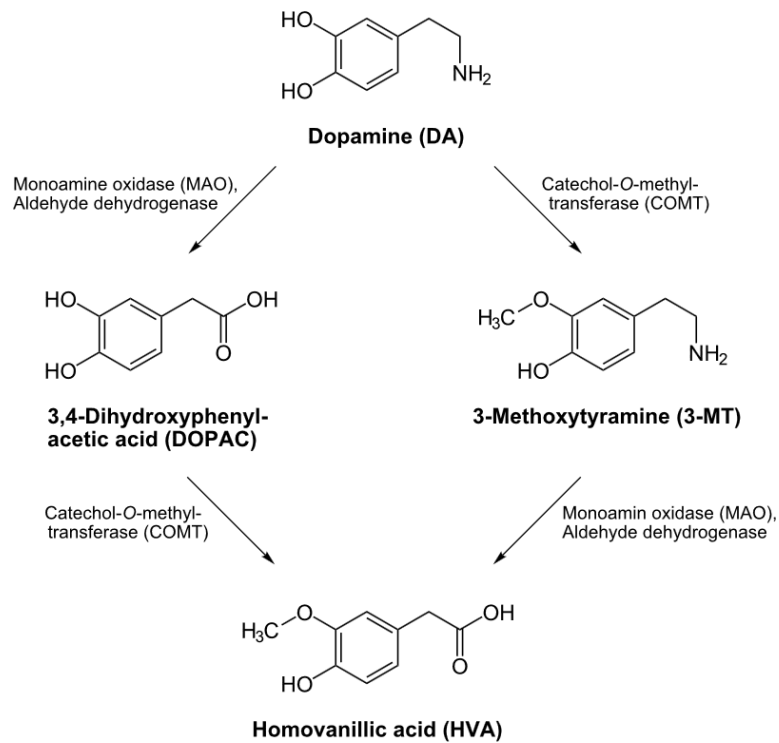


Fig.6. Degradation pathways of dopamine.

2.2. Dopaminergic receptors

Dopamine receptors belong to the family of G protein-coupled receptors (GPCRs) and, according to the type of associated protein, they are divided into two classes. The first includes the D1 and D5 receptors, which are Gs protein-coupled receptors and mediate excitatory effects by stimulating the production of adenosine monophosphate (cAMP) and phosphatidylinositol diphosphate. The second class includes D2, D3 and D4 receptors, which are Gi protein-coupled receptors and many effector systems, which include the deletion of Ca²⁺ flow and the activation of K⁺ flow, cause an intracellular decrease of these second messengers. Even structurally the two families have some differences: the D1 and D5 receptors have a long intracellular C-terminal tail, while the receptors D2, D3, D4, have in common the third intracellular loop very wide. At both central and peripheral level, the most relevant receptors are D1 and D2: centrally, they are the most abundant and widespread in areas that receive dopaminergic innervation (ie the striatum, the limbic system, the thalamus and the hypothalamus).

Depending on their localization, dopamine receptors can be divided into post and pre-synaptic receptors. The first are located on the dendrites, the cell body or on the nerve endings of not dopaminergic neurons (GABAergic, cholinergic, glutamatergic neurons, etc.). Presynaptic receptors are present instead on the nerve endings, dendrites and cell bodies of dopaminergic neurons. Receptors located on dopaminergic neuron are generically called presynaptic autoreceptors (Di Chiara et al., 1978). The physiological role of autoreceptors seems to be in order to prevent excessive activity of dopamine neurons. According to this hypothesis, when dopamine is released in excess by the dopaminergic neuron, it activates autoreceptors and inhibits the synthesis, the neurotransmitter release and electrical activity of the dopaminergic neuron. This effect is produced by dopamine released from dendrites. It has been demonstrated that the dopaminergic neuron releases dopamine not only from nerve terminals, but also from the varicosities of dendrites (Nedergaard et al., 1983).

2.3. Dopaminergic pathways in the CNS

In the central nervous system, cells that use dopamine as a neurotransmitter are easily recognizable by the lack of enzymes that convert dopamine into norepinephrine and then into epinephrine. Also thanks to these enzymatic characteristics, through electron microscopy and immunofluorescence techniques, it has been able over the years to identify dopaminergic pathways present in the brain. Dopamine in the brain is particularly abundant in the striatum, a component of the extrapyramidal motor system involved in the coordination of movements, and in some regions of the limbic system and the hypothalamus.

Cell bodies of dopaminergic neurons have primary location in the substantia nigra, in the ventral tegmental area (VTA), and in the hypothalamus where three main pathways originate:

- mesolimbic and mesocortical
- tuberoinfundibular
- nigrostriatal

The mesolimbic pathway includes mesencephalic fibers that project to:

- The nucleus accumbens, which together with the amygdala is part of the limbic system involved in emotional and cognitive behavior, and in regulating the sense of gratification;
- The olfactory tubercles, part of the limbic system, particularly developed in some animals for which the sense of smell is highly important.

The mesolimbic and mesocortical dopaminergic pathways are important, therefore, in brain processes of filtration of sensory stimuli, motivation of behavior and gratification as well as the psychotic effects caused by drugs of abuse.

The tuberoinfundibular pathway consists of fibers that originate from cell bodies placed in the arcuate nucleus in the hypothalamus and project towards the infundibulum interweaving with other fibers responsible for the control of prolactin release.

The third and final system is the nigrostriatal dopaminergic pathway, a key part of the extrapyramidal system responsible for the control of movement, posture and balance, and containing about 75% of dopamine in the brain. This pathway originates in the substantia nigra, a group of neurons located in the upper anterior midbrain, associated with the neurons of the striatum (Fig. 7). In Parkinson's disease these nigrostriatal fibers are affected by damage resulting in dopamine depletion.

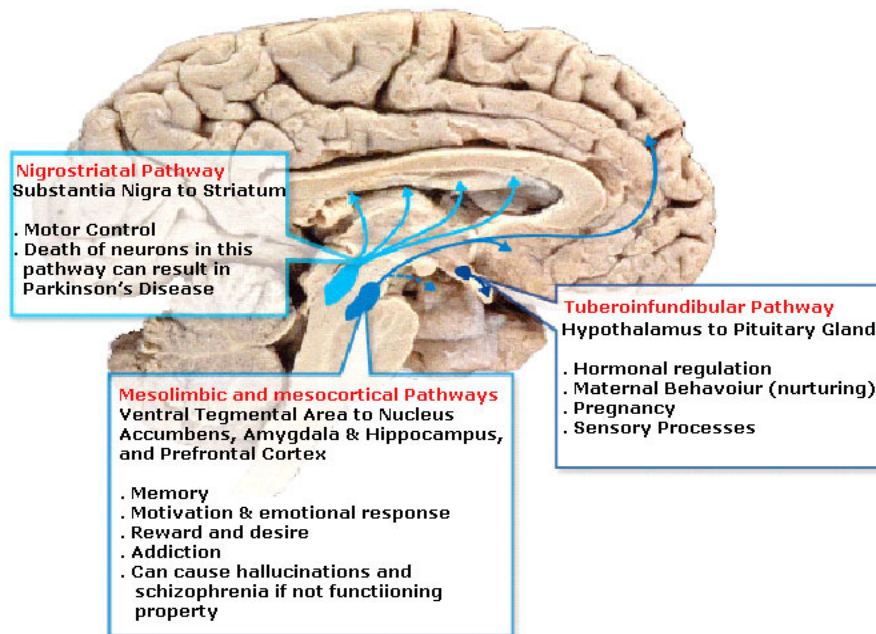


Fig.7. Central dopaminergic pathways.

The substantia nigra is a thin layer of gray matter consisting predominantly of dopaminergic neurons, whose name is due to the presence of high amounts of melanin pigment, which gives it a particular dark color. It is divided in two regions: *pars compacta* consists of nerve cells densely thickened and *pars reticulata* crossed by several bundles of myelinated fibers. Other afferents of the substantia nigra are represented by amygdala and the so-called raphe nuclei which are part of the serotonergic system. An efferent system from the substantia nigra is the nigrotalamic pathway which projects from the lateral thalamus to the primary and secondary motor areas of the cerebral cortex of the frontal lobe.

The connection between the striatum and the substantia nigra is organized in two main pathways: the **direct** and **indirect** pathways, with GABAergic modulation and

the resulting effect involves the activation or inactivation of the thalamus. The total excitatory or inhibitory effect comes from the balance between these two ways and is regulated by the action of dopamine released from the neuron terminals of the substantia nigra pars compacta (SN pc) on neurons of the striatum. D1 receptors mediate excitatory events, stimulating the excitatory direct pathway; D2 receptors mediate the inhibition of the indirect one, which reduces the excitatory flow from the thalamus to the cortex (Missale et al., 1998). Under physiological conditions the result of the balance of the two ways is a flowing and coordinated motion, but in Parkinson's disease, being there a deficiency of dopamine, the excitatory pathway is less stimulated and there is less control by the inhibitory pathway. The net result of these actions is a prevalence of the inhibitory signal to the thalamus, which translates to an uncoordinated movement and a lack of suppression of involuntary movements (Fig. 8).

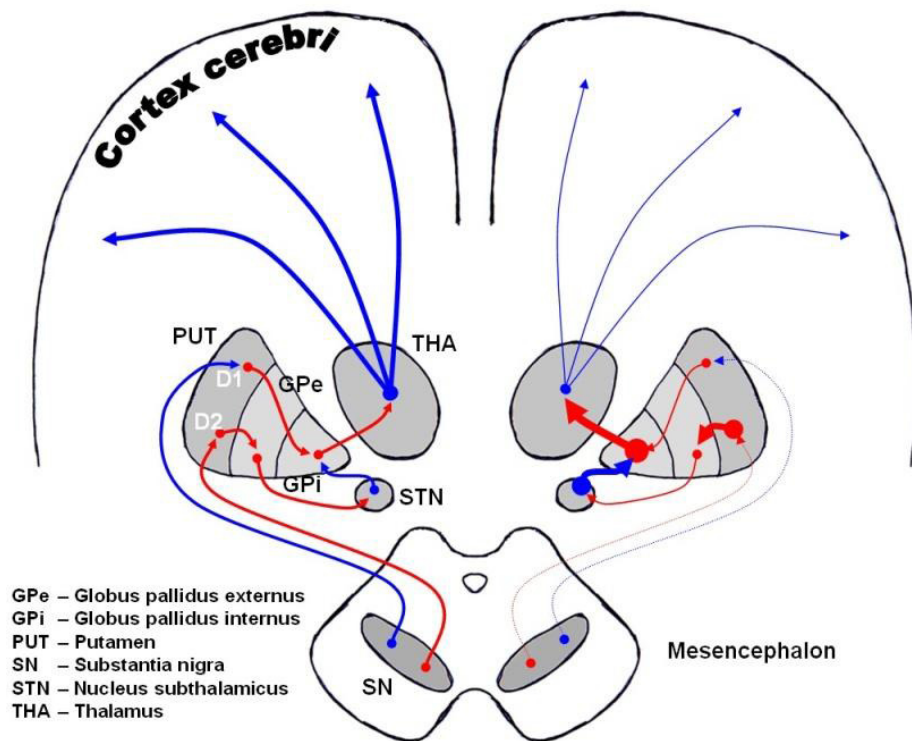


Fig.8. Representation of Nigrostriatal pathways (direct and indirect): in normal conditions (left) and in Parkinson's disease (right).

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3. Aetiology and pathogenesis

The aetiology of the degeneration of dopaminergic fibers is still quite unclear, however, from numerous experimental studies many are the causes that are not mutually exclusive, but rather mutually reinforcing in a vicious cycle of neuronal dysfunction, atrophy and finally, neuronal death.

About three decades ago the **hypothesis of viral disease** has been proposed, according to which it would be determined by the transmission of a virus, particularly lethargic encephalitis virus, responsible for postencephalitic parkinsonism. So far, however, this hypothesis has not found any confirmation (Lieberman et al., 2011). Occasionally, also, parkinsonian syndromes appear in the course of brain tumors in the frontal, mesencephalic, and parasagittal location (Nicholson et al., 1964): removal of the tumor resulted in some of these cases in a regression of symptoms. Currently, however, there are four main assumptions:

- 3.1. Toxic hypothesis
- 3.2. Genetic hypothesis
- 3.3. Multifactorial hypothesis
- 3.4. Theory of oxidative stress

3.1. Toxic hypothesis

It is already known that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Fig. 9), a contaminant synthetic of heroin and other opioid analgesics of abuse, can cause serious symptoms similar to those of Parkinson's disease.

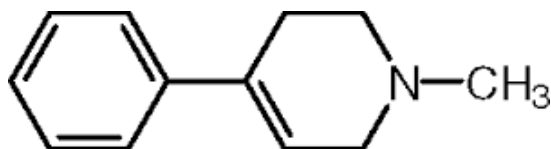


Fig.9. Chemical structure of MPTP

The toxic hypothesis asserts that continuous exposure to harmful substances, taken in small amounts, can cause injuries cause of the disease.

To become active, MPTP must be biotransformed in its toxic metabolite MPP⁺ (ion 1-methyl-4-phenylpyridinium), which is able to block the mitochondrial respiratory chain, directly inhibiting the electrons transport that allows the oxidation of NADH. This obviously converts into a decrease in the concentration of ATP with subsequent cell death. MPP⁺ can then be accumulated and stored in potentially toxic levels in dopaminergic terminals of the caudate nucleus, putamen (ie in the area of the striatum) and the pars compacta of the substantia nigra, entering into the cells via the dopamine transporter and destroying dopaminergic terminals (Fig. 10).

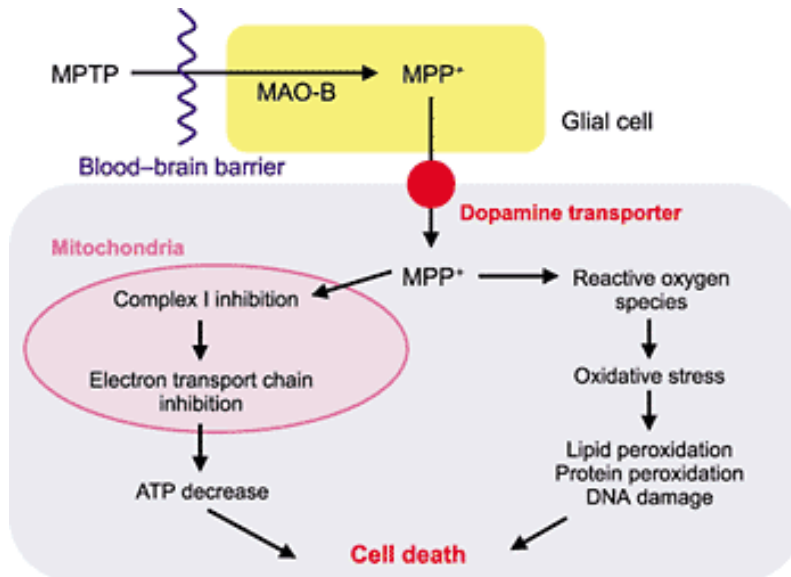


Fig.10. Toxicity mechanisms of MPTP.

It is also important to note that exposure to some pesticides correlates positively with the possibility of contracting Parkinson's disease: it was found that the combined exposure to fungicides and herbicides as Ziram, Maneb and Paraquat close to the workplace increases the risk of Parkinson's disease three times, while the combined exposure to Ziram and Paraquat has been associated with an increase of 80 per cent of the risk (*European Journal of Epidemiology, 2010*).

3.2. Genetic hypothesis

Parkinson's disease is a synucleinopathy with formation of Lewy's bodies in the cell body and Lewy's neurites in the extensions (axons-dendrites).

In recent years, several genes for monogenic hereditary forms of Parkinson's disease have been mapped and cloned. In a small number of families that have a typical pathology linked to Lewy's bodies with autosomal dominant inheritance, mutations have been identified in the **gene for the α -synuclein**. Aggregation of this protein in Lewy's bodies can be a crucial step in the molecular of familial and sporadic pathogenesis (Baba et al., 1998) of Parkinson's disease. On the other hand, mutations in the gene called *parkin*, cause autosomal recessive early-onset parkinsonism linked to mitochondrial dysfunction (Petruzzella, 2012). In this form of Parkinson's disease, the nigral degeneration seems not to be accompanied by the formation of Lewy's bodies.

The *parkin* gene expresses a protein involved in the degradation pathways of cellular proteins, it has been shown that it works as a ubiquitin ligase (Gasser, 2001). The potential importance of this pathway is also highlighted by the discovery of a mutation in the gene that expresses a ubiquitin C-terminal hydrolase L1, in another family affected by the disease.

Parkin mutations therefore appear to be a common cause of Parkinson's disease in patients with early onset. These results show that there are several genetically distinct forms of Parkinson, which can be caused by mutations in single genes. On the other hand, however, there is currently no evidence that these genes play a direct role in the aetiology of common sporadic form of Parkinson.

In the end, case-control epidemiological studies, even if they support a genetic contribution to the development of Parkinson's disease, suggest that a clear family origin is only present in a minority of cases (Gasser, 2001).

3.3. Multifactorial hypothesis

To date, one of the most accredited etiological hypotheses is the one that identifies a multifactorial genesis of the disease, including a hereditary component and exposure to certain environmental factors (Silva, 2000).

The results of recent molecular studies delineate a sequence of pathological events where deficit of synaptic exocytosis, lysosomal mediated-autophagy and mitochondrial stress increase susceptibility to Parkinson's disease (Trinh, 2013). The **multifactorial hypothesis** is supported by the fact that exposure to toxic substances, genetic alterations and dietary habits are all positively correlated with the onset of the disease (Costello et al., 2009). People with Parkinson's disease have genetically reduced capacity for detoxification, resulting therefore particularly vulnerable to the cytotoxic effects of substances or highly reactive metabolites which have also reached the central nervous system. Among the environmental and occupational factors that may increase the risk of developing the disease it is included exposure to exogenous toxins such as pesticides, metals, industrial chemicals, lifestyle (diet and smoking) and place of residence (rural) (Costello et al., 2009).

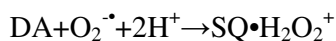
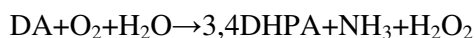
Even dietary habits can affect the manifestation of Parkinson's disease. It is well known indeed that a deficiency of vitamin D has positive effect on the development of the disease, while foods such as nuts, legumes, potatoes and generally caffeine, seem to play a protective role (Evatt et al., 2008).

3.4. Theory of the oxidative stress

The human brain is only 2% of body weight, but consumes an amount of oxygen far superior to that of all the other organs (about 20% of the available quantity). The high brain metabolic activity, combined with the reduced ability of cell regeneration and the lower concentration of catalase, glutathione peroxidase and tocopherols, makes the structures of the central nervous system (CNS) particularly susceptible to oxidative damage. The oxygen then assumes the dual role of vital importance and potential toxicity. The **oxidative damage** is to be considered as a consequence of an alteration of the balance between pro- and cellular antioxidant activities.

Dopaminergic neurons are particularly vulnerable to oxidative stress, since dopamine metabolism generates hydrogen peroxide (H_2O_2) and other reactive oxygen species (reactive oxygen species, ROS) such as superoxide radical ($O_2^{\cdot-}$), the radical hydroxyl (OH^{\cdot}) and singlet oxygen (1O_2) (Olanow, 1993).

Dopamine in neurons, in addition to the metabolic enzymatic pathways, can be subject to a non enzymatic self-oxidation in semiquinone radical (\cdot SQ) according to the reactions:



The H_2O_2 and $\text{O}_2^{\cdot-}$ are normally eliminated by enzymatic reactions involving the superoxide dismutase (SOD), glutathione peroxidase (GPO) and catalase. A constant concentration of H_2O_2 accompanied by a significant increase of Fe^{+2} can lead to the Fenton reaction with formation of the radical OH^{\cdot} .

The mitochondria of patients with Parkinson's disease have several abnormalities. The most documented is the decrease of activity of Complex I: the inhibition of this enzyme complex involves the reduction of ATP production, an increase of dispersion of electrons, and an increase of the production of superoxide and hydroxyl radicals. This could be behind the increase of oxidative stress associated with the disease (Petrovitch et al., 2002).

Thus oxidative stress is the result of radical overproduction or deficiency of antioxidants. In such circumstances the reactions in lipids, proteins and nucleic acids can cause irreparable damage that leads to cell death. There are several sources that generate radicals; between these depletion of glutathione, the mitochondrial dysfunction, accumulation of metals and the same oxidative metabolism of dopamine leading to the formation of highly reactive products (Spencer et al., 1996; Spina et al., 1989). In particular, it was recognized that metal concentrations are elevated in the substantia nigra of patients with PD (Sayre et al., 2005; Jellinger et al., 1992).

One of the more deleterious consequences of oxidative damage is lipid peroxidation whose marker, malondialdehyde (MDA), the final product of the oxidation of polyunsaturated fatty acids, is evident in the substantia nigra of PD patients (Dexter et al., 1989; Dexter et al., 1994, Dexter et al., 2006), in the cerebrospinal fluid (Shukla et al., 2006) and in plasma and urine (Kikuchi et al., 2003). Similar studies were also carried out on models of Parkinson's disease by 6-hydroxydopamine (6-OHDA) in rats and the results show high levels of MDA in the striatum (Kumar et al.,

1995; Tuncel et al., 2012), in the temporal lobe (Ciobica et al., 2012), heart and plasma (Talanov et al., 2009).

Moreover, the hypothesis of free radicals has received support from post-mortem studies on brains of patients: there is clear evidence of the damage caused by free radicals in all cellular structures mentioned above (Zigmond et al., 2002).

4. Symptoms of the disease

4.1. Motor symptoms

In Parkinson's disease the loss of dopaminergic neurons in the substantia nigra affects the ability of the basal ganglia to coordinate excitatory and inhibitory motor signals (Kwan & Whitehall, 2011). The net effect is an overall reduction of motor response or Hypokinesia. The drugs used to treat the disease may introduce too much dopamine, causing hyperactivation of the motor system and producing dyskinesia (fragmented movements, spasms, or tics). The motor symptoms associated with Parkinson's disease adversely affect all aspects of a daily activity, balance, postural stability and mobility (Robertson L., 2013).

➤ Tremor

Parkinsonian tremor is characteristically a tremor at rest, which is temporarily interrupted by voluntary movement. This symptom typically accentuated by emotions, increases especially if patients feel to be observed: It is not unusual for these, once came to visit the doctor, claim to be suddenly worsened. With the will it is possible for the subject to stop shaking for a few seconds, and then see it reappear more intense: during the voluntary suppression in a limb, it becomes more pronounced in other districts. At the beginning of the disease, the tremor may be poorly detectable, more palpable than visible. Usually it increases with the course of time, in a smaller number of patients decreases with the increase of rigidity.

With the progress of the disease tremor extends to the jaw, lips, tongue and head: in this case it seems that the patient "is performing an endless litany." Sometimes with

the laryngoscope is possible to detect a rhythmic tremor also in the vocal cords, adjustable by respiratory movement and phonation (Introzzi, Italian Essay of Internal Medicine, 1984).

➤ Stiffness

Parkinsonian rigidity, ie the increased muscular opposition to passive movement, affects the entire execution of the movement, giving the examiner the impression of an excessive plasticity. Unlike the tremor, rigidity often affects more the axial muscles (neck, trunk, root of the limbs), however all body segments may be interested. Such as tremor also the rigidity, especially at the beginning of the disease, is sometimes limited to a limb or to a side of the body, sometimes being more marked on the side opposite to that where is present the tremor. The input of tremor on stiff muscles gives rise to the phenomenon of the “toothed wheel”, consisting of a succession of contractions and relaxations during the passive motion of a joint. The phenomenon, as has been said, is the result of the coexistence of rigidity and tremor in the same muscle for which passive movements meet a fluctuation of resistance. Although tremor and rigidity are kept separate, they are the expression of the contractile process in the same muscle group (Mutani, The Bergamini of Neurology, 2011).

➤ Akinesia

It represents one of the most prominent characteristics of the disease, and consists of an impaired ability to initiate motor activity, whether intentional or spontaneous: the motor system seems to lack spontaneity and leadership, the arts seem lazy (Angel et al., 1970). Akinesia clinically manifested by the amimia, which gives the patient the characteristic "Parkinsonian mask": the features are fixed, immobile look, rare facial expressions. At the same time automatic movements or associated are reduced or eliminated: the lack of oscillation of the arm in walking is a characteristic sign and premature. Even the intermittent automatic swallowing movements tend to disappear, so what follows is an accumulation of saliva in mouth, with apparent drooling.

The hesitation in starting a movement is the cornerstone of the disorder: the patient appears "reluctant" to begin an activity, while its later stages generally take place with less difficulty. Typical in this regard is the difficulty to start walking: once he set in motion the parkinsonian patient can walk with relative easiness, sometimes accelerating the steps until run. As the disease progresses, the execution of the movement becomes more difficult.

Associated to akinesia is bradykinesia (slowness in movement) and the reduction in amplitude of the movements themselves. It results for example in the characteristic micrographia and the difficulty to stride. The akinesia of a parkinsonian has a different meaning than tremor and rigidity, which is independent. These last two are called positive symptoms, ie due to abnormal discharges in the motor system, while akinesia, like the postural disorders, is a negative symptom (Introzzi, Italian Essay of Internal Medicine, 1984).

4.2. Non-motor symptoms

- Gastrointestinal disorders

Among the autonomic disorders, complications in the gastrointestinal system are the most peculiar. Dysphagia, drooling, feeling of nausea, bloating and constipation are all common symptoms of Parkinson's disease. The same dopamine changes in brain that cause stiffness and slowness of movement, also affect the muscles involved in swallowing and progression of the bolus of food through the digestive tract. In addition, Parkinson's disease can negatively affect the enteric nervous system. Gastrointestinal symptoms begin to occur on average about ten years before the onset of motor symptoms that characterize Parkinson's disease (Abbott et al., 2001). These symptoms get worse as the disease progresses. Dysphagia was found in 50-90% of patients (Edwards et al., 1994), while constipation is caused by a delayed colonic transit, a condition which is observed together with gastroparesis in more than 50% of cases (Winge et al., 2003). Anorectal manometry, electromyography and defecography showed impaired coordination of contraction and relaxation of the

abdominal-pelvic and pubo-rectal muscles with impaired function of the anal sphincter (Jost et al., 1998).

➤ Dysphagia

Dysphagia in Parkinson's disease generally originates from alteration of all three phases of swallowing (oral, pharyngeal and esophageal) that are controlled at different levels by a somatic mechanism, but also by the autonomic nervous system. Alterations of lingual and buccal motility are often present in patients with consequent difficulty in bolus formation and swallowing. The main functional abnormalities in pharyngeal level, as a result of bradykinesia and muscular rigidity, consist of a reduced upper esophageal sphincter relaxation and an altered functioning of the cricopharyngeal muscle (Dachshunds et al., 1998; Ali et al., 1996; Williams et al., 2006). Numerous dysfunctions have been described that cause a difficult progression of the bolus including slowed oesophageal transit, segmental and diffuse esophageal spasm, non-peristaltic contractions, repeated contractions in proximal esophagus, esophageal dilatation and reduced pressure at the lower esophageal sphincter (Pfeiffer, 2003; Castell et al., 2001; Dachshunds et al., 1998). The disease is therefore characterized by progressive loss of esophageal peristalsis and the inability to relaxation of the lower esophageal sphincter (LES), located on the border between the esophagus and stomach (Trugman et al., 2008). Dysphagia is one of the clinical symptoms that does not seem to be correlated with the severity or duration of the disease (Ali et al., 1996) and it can also cause aspiration pneumonia, a bronchopneumonia that develops as a result of the entry of strange materials in bronchial tree.

➤ Gastroparesis

Regarding the functionality of gastric motility, the rate of gastric emptying is regulated by multiple mechanisms that allow the transfer of the chyme in the duodenum at a speed appropriate to its digestive capacity: in these processes, the contractions of the gastric body and the coordinated motor events of antrum, pylorus

and duodenum are essential. When these functions are altered, there is a delayed stomach emptying named gastroparesis.

In patients with Parkinson's disease this symptom generally gives rise to several symptomatic manifestations such as postprandial bloating, early satiety, nausea, vomiting, causing eventually weight loss and malnutrition (Jost et al., 1997; Pfeiffer, 2003, 2011; Hardoff et al., 2001). Moreover, this phenomenon can lead to serious problems in the therapeutic management since the drugs effect is significantly delayed (Del Tredici et al., 2012). Patients often complain of indigestion and heartburn, caused by reduced peristalsis or a gastroesophageal reflux, most likely due to gastric and esophageal dysmotility and LES dysfunction (Ali et al., 1996; Pfeiffer, 2003). At different stages of the disease it was reported a progression of the delay of gastric emptying time (Hardoff et al., 2001; Melamed et al., 1999; Djaldetti et al., 1996): the EGG (electrogastrography, or the study of electrical activity of the stomach) showed an altered gastric motility both in early and in late disease (Pfeiffer, 2003; Soykan et al., 1999; Krygowska-Wajs et al., 2000).

➤ Constipation

Even the normal intestinal physiology can be compromised: in the small intestine there is a variation of the motility which can result in abdominal distension (Bozeman et al., 1990). The coordinated actions of the rectum, anal sphincter and abdominal muscles determine the expulsion of fecal material: altered colonic motility frequently causes constipation, defined as reduced bowel movement (two or fewer bowel movements per week) which is associated with difficult and inadequate evacuation (Pfeiffer, 2003; Jost et al., 2003). Constipation is a common symptom in patients with Parkinson's disease, with a frequency of around 80% (Verbaan et al., 2007; Dachshunds et al., 2000; Kaye et al., 2006; Siddiqui et al., 2002) , with a transit time more than doubled compared to control subjects. Constipation can occur at all stages of the disease, even before the onset of motor symptoms (Chaudhuri et al., 2005; Verbaan et al., 2007). Constipation, in the later stages of the disease, can be wrongly considered as a side effect of dopamine replacement therapy: even patients who are not undergoing any drug treatment have a delayed colonic transit and constipation (Abbott et al., 2001; Verbaan et al., 2007), suggesting that this symptom is related to a

direct involvement of the motility of the colon and not (or not only) to the side effects of therapy (Pfeiffer, 2003). Some studies have found that constipation increases with the progression of the disease itself (Verbaan et al., 2007). The intrinsic neurons of the colon are often involved, as indicated by the presence of Lewy's bodies in the myenteric plexus (Kupsky et al., 1987; Jost et al., 1994) and as evidenced by the fact that the loss of these neurons can lead to colonic inertia (Pfeiffer, 2003).

- Micturition disorder

Urinary tract dysfunction can occur in patients with Parkinson's disease in the form of an urgent need to urinate, changes in the frequency of micturition or difficulties in initiating or completing it. These disorders seem to be associated with the rigidity of the pelvic muscles that could be an obstacle to bladder function. Several studies have shown that among the various dysfunctions of the urinary tract, the most common are: problems of bladder filling (57% - 83% of patients), bladder voiding (17% - 27%), urgent need to urinate (33% - 54%) and nocturia (over 60%) (Yeo et al., 2012). The alterations of urination were attributed to a modified involvement of the various subtypes of dopamine receptors in the district. The D1 family receptors inhibit, while those of the D2 family facilitate the micturition reflex. Based on the influence of D1 and D2 agonists and antagonists on the bladder emptying, Seki et al. concluded that such dysfunctions are related to a failure in the activation of D1 receptors and an overactivation of D2 receptors (Seki et al., 2001).

- Depression

A state of depression occurs in about half of patients with PD (C. Adler, 2005). In these subjects depression precedes Parkinson's disease and sometimes turns out to be more debilitating than the disease itself. The serotonergic antidepressant agents (fluoxetine, sertraline, paroxetine and fluvoxamine) block the neuronal transport of serotonin. The increase in availability of the latter at the synaptic level stimulates a large number of 5-HT receptors; stimulation of the 5-HT₃ contributes to the

occurrence of adverse effects such as vomiting and nausea while stimulating 5-HT₂ receptors may contribute to the onset of crisis of agitation and restlessness. Even tricyclic antidepressants (TCAs) are effective, but the frequent anticholinergic effects and orthostatic hypotension restrict its use in patients with PD and depression. The use of TCA, which have sedative properties, thus tends to be limited to a low dose intake at bedtime at night, in order to assist in the treatment of sleep disorders.

- Sleep disorders

Sleep disorders are common among patients with PD and include: difficulty in falling asleep at night, nights with frequent awakenings and daytime sleepiness. These can also occur as side effects as consequences of drug treatment (C. Adler, 2005).

- Mental and cognitive alterations

The cognitive alterations appear late in the course of the disease (after a few or several years of onset) and include impairment of memory, of the ability of abstraction, calculation and recognition (C. Adler, 2005). Parkinson's disease without dementia usually begins in 40-60 years old patients, it has a long course, and responds well to medication; in the form with dementia begins in older people and usually has a shorter and more severe course.

5. The importance of the Enteric Nervous System in Parkinson's disease

5.1. Neurotransmitters and neuromodulators

The Enteric Nervous System (ENS) is a real miniature nervous system located in the wall of the gastrointestinal tract. A neural network controls reflex movements of various sections of the intestine, although the latter is isolated from the rest of the body. The afferent neurons, interneurons and motor neurons are included in the SNE,

whose activity is regulated by the sympathetic and parasympathetic afferent systems. The component of the SNE present in the myenteric plexus of Auerbach controls the activity of the muscle layers, while the one located in the submucosal plexus of Meissner controls the activity of the muscularis mucosae and intestinal glands. The connection to the central nervous system is guaranteed by the vagus nerve and the pre-vertebral sympathetic ganglia (celiac, lower and upper mesenteric) (Fig. 11).

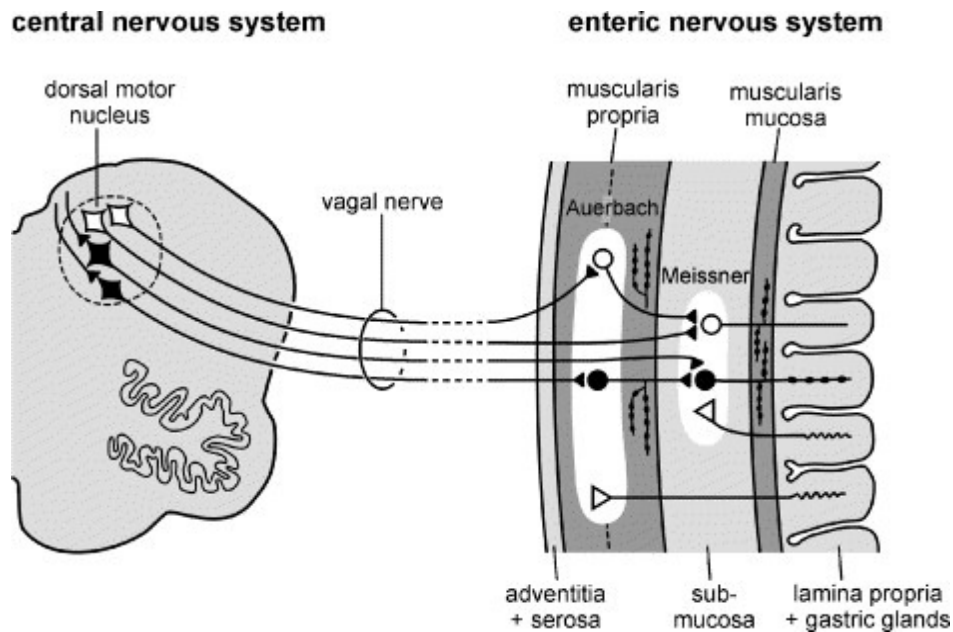


Fig. 11 Schematic diagram of the interconnections between the enteric nervous system and brain. Taken from Braak et al., 2006.

These plexuses are responsible for gastrointestinal peristalsis (Djaldetti et al., 2009). In both plexuses of patients with Parkinson's disease Lewy's bodies have been identified, most frequently in the lower part of the esophagus (Beach et al., 2010; Wakabayashi et al., 1988). The synucleinopathy was also highlighted in the submandibular glands, stomach, small intestine, colon and rectum in PD patients. Motor neurons that release the inhibitory vasoactive intestinal peptide (VIP) and receiving preganglionic fibers of the vagus nerve seem particularly altered in Parkinson's disease (Braak et al., 2008). The raphe nuclei are equally involved, with consequent alteration of the supraspinal control of defecation (Jain, 2011).

Cholinergic, noradrenergic, serotonergic, purinergic and peptidergic neurons can be recognized in the myenteric plexus.

The **cholinergic neurons** of the myenteric plexus exert excitatory action on gastrointestinal motility. Cholinergic transmission is present at various levels: on the esophageal striated muscle there is vagal innervation, the ascending interneurons as well as the motor secretory and vasomotor neurons are cholinergic (Furness, 2000).

Adrenergic neurons, numerous in both myenteric and submucosal plexuses, have inhibitory action on gastrointestinal motility. It is supposed that the releasing action of noradrenergic neurons does not take place for a direct action on the smooth muscle of the mediator, but through inhibition of excitatory neurons.

Serotonergic neurons release 5-hydroxytryptamine, which activates excitatory cholinergic neurons, but also inhibitory noradrenergic neurons in both myenteric plexus and submucosal plexus. It is therefore considered that serotonergic neurons do not act directly on the muscles, but instead play modulatory action on other neurons of the enteric nervous system.

Purinergic neurons have inhibitory action on gastrointestinal musculature. It has been demonstrated that the ganglionic neurotransmission in the guinea-pig ileum in the myenteric plexus includes NANC (non-adrenergic / non-cholinergic) synapses since a portion of this neurotransmission can be inhibited by suramin (a purinergic receptor antagonist) (Lepard et al., 1997). These neurons then would perform their function through the release of ATP, which is a potent inhibitor of gastrointestinal motility.

Peptidergic neurons release polypeptidic neurotransmitters as vasoactive intestinal peptide (VIP), somatostatin, enkephalins that appear to play a direct inhibitory action on muscles, and stimulating inhibitory neurons or inhibiting cholinergic neurons.

The already mentioned intestinal motility disorders in Parkinson's disease, such as constipation, have been attributed to the presence of damage in the enteric nervous system or at the level of the fibers that integrate the control of intrinsic enteric system

with the extrinsic one (Lebouvier et al., 2009). The sympathetic denervation was indeed associated with an increased expression of n-NOS in the enteric nervous system and this has suggested that the extrinsic nervous system influences the production of NO (Nakao et al., 1998; Yunker et al., 1998).

Even peptidergic neurons are altered in patients with Parkinson's disease. Studies performed in rats with pharmacologically induced PD show changes in bowel function and expression of certain enteric neurotransmitters: a decrease of nitrergic neurons and a corresponding increase in the synthesis of VIP (Colucci et al., 2012); this compensatory phenomenon is not, however, able to restore normal intestinal motility.

Constipation and other abnormalities of gastrointestinal function, characteristic of PD patients and sometimes reproduced in animal models of Parkinson's disease, have been attributed to disruptions in both intrinsic and extrinsic nervous system, in particular to the decrease of D2 receptors (Blandini et al., 2009).

5.2. Interstitial Cells of Cajal (ICC)

As already mentioned above, gastrointestinal dysmotility may be caused by changes in the neural and/or myogenic control systems resulting in an altered progression of intraluminal contents. An essential component of the myogenic control is represented by the electrical pacemaker activity that originates in the interstitial cells of Cajal (ICC). ICC are pacemaker cells for many rhythmic motor activities throughout the gut. The first modern paper to promote the concept of ICC as pacemaker cells was written in Italian but is now available as English translation (Faussonne-Pellegrini MS et al. 2013).

Several ICC sub-types have been identified. One population of ICC (the ICC at the myenteric plexus of Auerbach, called ICC-MP or ICC-AP or IC-MY) is located at the ganglionated myenteric plexus level all along the gut length and has been identified as the source of the electrical slow wave-driven peristalsis, which is prominent during gastric emptying and small bowel transit; another ICC population is located intramuscularly and exists as two ICC sub-types: those located within muscle bundles are called intramuscular ICC or ICC-IM and mediate excitatory and inhibitory inputs to the musculature from the enteric motor neurons, and those located in the septa

dividing muscle bundles are called ICC-SEP (Fig.12); A third ICC population (the ICC-SMP or ICC-SM) is distributed along the submucosal border of the circular muscle layer of the gastric antrum and the colon. Finally, a fourth ICC population (the ICC-DMP) is present in the small intestine, distributed between the inner and the outer portions of the circular muscle layer at the level of the deep muscular plexus (DMP). All the ICC sub-types have in common to be closely associated with nerve endings, with each other and with smooth muscle cells (Fausone-Pellegrini MS 2006).

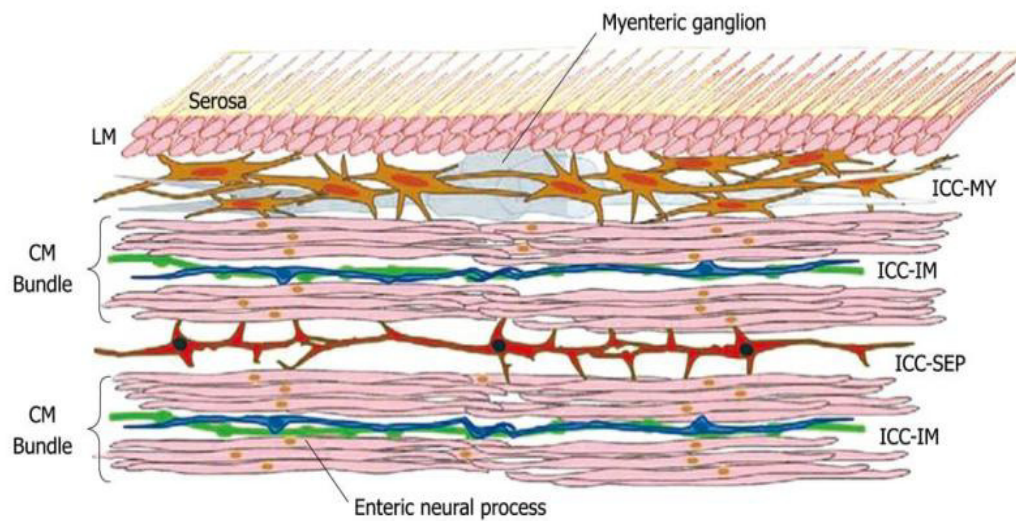


Fig. 12. ICC subtypes

ICC harbor the c-Kit protein and can therefore be identified at the light microscopy level using immunohistochemistry with c-Kit antibodies. In fact, almost all current studies on the pathology of ICC are performed by immunostaining (Wang XY et al, 2002).

Considering the importance of these cells, their role and how gastrointestinal motor activity is altered in case of their structural change or damage led us to investigate whether GI motility disorders in Parkinson's disease can be attributed to alterations of this type of cells.

6. Current therapeutic treatment

A common cause of parkinsonism in 1920 was recognised in encephalitis, which prompted an attempt to develop a vaccine that would prevent the development of postencephalitic parkinsonism. This earlier effort to develop protective immunologic therapy, has now been replaced by trials investigating agents that may represent neuroprotective therapy. Potential examples include coenzyme Q10 (antioxidant and mitochondrial stabilizer) and minocycline (anti-inflammatory/antiapoptotic). This growing recognition of the mechanisms and pathways potentially involved in the death of dopaminergic neurons in PD is leading to an ever-expanding array of investigative approaches whose aim is to achieve effective neuroprotective or even neurorestorative treatment. Avenues being investigated encompass not only traditional (and non traditional) pharmacological approaches, but also innovative and frontier-crossing surgical and other modalities such as gene therapy, stem cell therapy, and neurotransplantation (Ebadi et al., 2005).

To date there is still no cure for Parkinson's disease, but both pharmacological treatment and surgery are able to provide relief especially to the motor symptoms.

The stage of the disease determines which category of drugs is more useful. The rationale of the therapy involves restoring the deficiency of dopamine through the administration of dopamine-mimetic (classified according to their mechanism of action) and reduced cholinergic activity through the administration of anticholinergics. The treatment in the initial phase with the MAO-B inhibitors and dopamine agonists delay the use of levodopa hoping to delay the onset of dyskinesias. The current therapeutic strategy continues to be symptomatic and when the drugs are no longer sufficient to control the symptoms, surgery and deep brain stimulation may be useful.

6.1. Pharmacologic therapy

- L-Dopa and Dopaminergic agonists

Treatment with L-Dopa (Levo-dihydroxyphenylalanine Levodopa) is currently the most effective device available, although more than thirty years have passed since its introduction into the trade.

Its therapeutic activity as well as its side effects result from its decarboxylation to dopamine. After oral administration, levodopa is rapidly absorbed from the proximal small intestine, using the transport system of the aromatic amino acids. The transit of the drug into the brain through the blood brain barrier is an active process mediated by the aromatic amino acid transporters. In both cases, there may be competition with the amino acids taken in with diet. In commercially available preparations, levodopa is associated with an inhibitor of dopa decarboxylase (ie benserazide, carbidopa) to prevent the peripheral conversion to dopamine. These inhibitors increase the bioavailability of levodopa, allowing to reduce the dosage and peripheral side effects. The drug improves considerably all parkinsonian symptoms and is equally effective on tremor, rigidity and akinesia. However, it is worthy to note that, with the evolution of the disease, doses become less effective, probably because the dopaminergic terminals continue to degenerate losing the ability to effectively respond to the action of exogenous L-dopa.

Dopamine agonists (ie apomorphine, ropinirole) bind directly to the postsynaptic dopamine receptor and mimic the effect of dopamine.

Dopamine agonists in addition to levodopa therapy have been introduced for the treatment of advanced stages of Parkinson's disease, but it has been demonstrated their efficacy in monotherapy also in the early stages of the disease, particularly early onset, in order to delay the development of motor fluctuations (Sghirlanzoni, therapies of neurological diseases, 2010).

Functional neuroimaging has allowed to study the possible neuroprotective mechanism of some dopamine agonists: subjects treated with dopamine agonists maintain higher dopaminergic neuronal functionality than controls during monotherapy with levodopa.

- Dopaminergic re-uptake inhibitors

Amantadine is an antiviral drug whose action with favourable effects on parkinsonism was discovered by accident in patients that were taking it to prevent flu. The mechanism of action is unclear: it seems that amantadine results in an increase of usable extracellular dopamine, interfering with the recovery phase of the latter. It should, however, be underlined that adequate evidence of the therapy support have not yet been demonstrated (Crosby et al., 2003).

- Inhibitors of dopamine catabolism

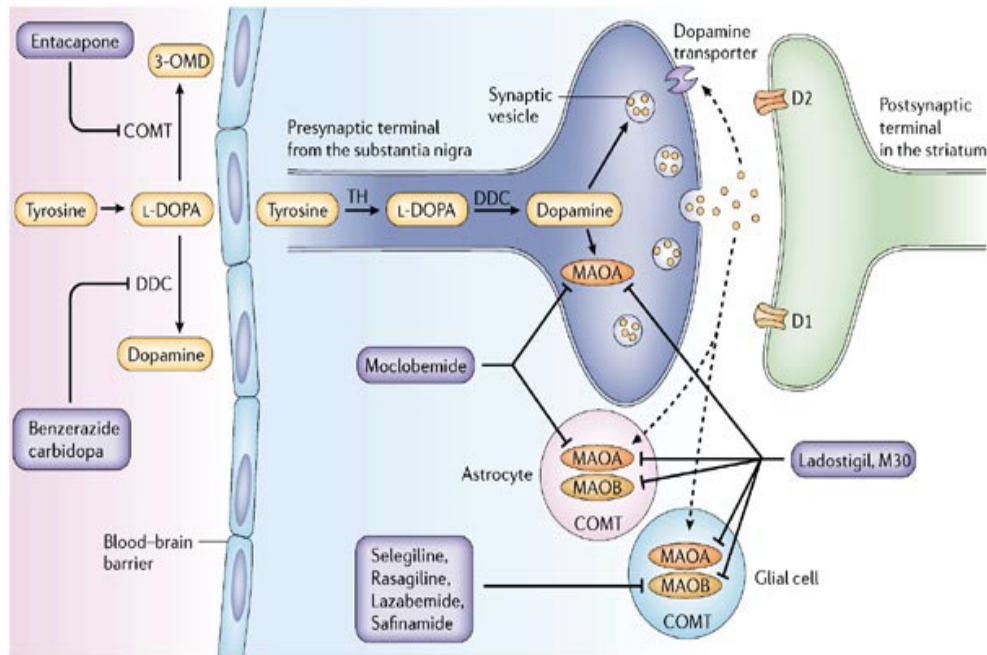
This category of drugs inhibit enzymes involved in the catabolism of catecholamines: MAO-B and COMT.

Selegiline is a selective inhibitor of MAO-B which predominates in the CNS regions containing dopamine, and protects intraneuronal dopamine from degradation. Selegiline, in addition to inhibition of MAO-B, blocks the re-uptake of dopamine and norepinephrine in presynaptic nerve terminals and increases dopamine turnover. Entacapone is a peripheral inhibitor of catechol-O-methyltransferase (COMT), an enzyme that transfers a methyl group on the catechol on levodopa inactivating it. Its combination with levodopa / carbidopa is exploited in those patients suffering from motor fluctuations (rigidity and hypokinesia) of "end of dose".

- Anticholinergic drugs

For many years anticholinergic drugs have been used as first-line treatment in Parkinson's disease: it is known the presence on corpus striatum, of cholinergic innervation (which predominates when the dopaminergic transmission is depressed, as in Parkinson's disease).

Use of cholinergic receptors antagonists (specifically antimuscarinic) as procyclidine, orphenadrine, biperiden and trihexyphenidyl, compensate for this transmission imbalance: centrally attenuate tremor and rigidity, while peripherally reduce incontinence and drooling .



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Fig. 13 Therapeutic treatment strategies

6.2. Surgical therapy

Studies of recent decades have led to major improvements in surgical techniques, with the consequence that surgery is again used in people suffering from PD and when drug therapy is no longer sufficient.

Deep brain stimulation (DBS) is the most commonly used surgical treatment and allows a good clinical remission and a significant reduction in dependence on Levodopa. It involves implanting of a medical device, a brain pacemaker, which sends electrical impulses to specific areas of the brain. DBS is recommended for patients who suffer from strong tremor not adequately controlled by medication or in those who are intolerant to drug treatment (Bronstein et al., 2011).

6.3. Innovative therapy

Gene therapy involves the use of non-infectious viruses engineered with the gene encoding the protein of GABA synthesis that is deficient in patients suffering from

the disease. The virus containing the gene is injected into the subthalamic nucleus, which regulates the motor circuit.

In 2010 there were four clinical trials that had used gene therapy in PD. There were no important adverse effects in these studies, although the clinical utility of gene therapy has still to be determined (Obeso et al., 2010).

Stem cell therapy

Since 1980, transplants of pluripotent stem cells induced in the substantia nigra, have been tried hoping to replace degenerated nerve cells and to resume production of dopamine. Despite expectations based on initial studies, more recent studies have not shown any long-term benefit (Obeso et al., 2010).

7. Experimental models for the study of Parkinson's disease

Modern research can use experimental models of Parkinson's disease, based on the use of different animal species, which continue to provide valuable information, while also allowing to experiment new therapeutic strategies.

Based on the techniques used to reproduce the histopathologic and functional characteristic of the disease, animal models of Parkinson's disease can be basically divided into two categories: pharmacological and transgenic (Blandini, 2004).

7.1. Pharmacological models

- 6-OHDA and MPTP

The pharmacological models of Parkinson's disease are the most used by far and are based on the reproduction of the pathologic lesion typical of the disease, the degeneration of dopaminergic neurons of the substantia nigra, induced by administration of specific neurotoxins (systemic or local) (Blandini, 2004). These models are based essentially on the use of primates or, more diffusely, of rodents.

In primates, the lesion is produced by intracarotid injection of MPTP. The MPTP, once crossed the blood brain barrier, is transformed by monoamine oxidase (MAO) in its active metabolite, MPP⁺. It accumulates selectively at the level of dopamine

neurons, using the transport system for dopamine and binds to the complex I of the mitochondrial respiratory chain, causing cell death. In monkeys, this procedure causes onset of parkinsonism that is, from the point of view of symptoms, the experimental form of Parkinson near to human idiopathic disease (DeLong, 1990).

The majority of studies on the experimental Parkinson's disease, however, is conducted on rodents, basically for cost reasons. Analyzing the scientific literature of the last decade, it shows that about 80% of these studies is conducted in rats (in most cases) and mice.

The absolutely most used model is based on the use of 6-hydroxydopamine (6-OHDA), a specific toxin for dopaminergic neurons that causes cell death through the induction of oxidative stress and apoptosis (Blum et al., 2001; Ungerstedt et al., 1974).

6-OHDA is infused stereotaxically, usually at the level of the substantia nigra or in the bundle that contains the nigro-striatal projections (defined medial forebrain bundle -MFB), causing a rapid and almost complete in situ depletion of dopamine neurons. The toxin can also be infused into the striatum, ie at the level of the nigro-striatal terminals: in this case, the lesion of cell bodies in the substantia nigra occurs in a retrograde way, subsequently to the terminal damage. The injury is minor, compared to the infusion of 6-OHDA, and takes much longer time for establish, reaching the peak in 2-3 weeks (Sauer et al., 1994).

The gradual evolution of lesion thus leaves an open therapeutic window, exploitable to evaluate the effectiveness of treatments aimed to oppose the progression of nigrostriatal damage due to 6-OHDA (Deumens et al., 2002).

Other pharmacological models of Parkinson's disease in rodents, used less frequently, include systemic administration of MPTP or reserpine (dopamine consumer) in the mouse (Kaakkola et al., 1990) or the direct infusion of MPP + in the substantia nigra or in striatum of the rat (Srivastava et al., 1993). The direct use of MPTP systemic route, in the rat, is compromised by the poor sensitivity of this species to the toxin. The reasons for this reduced susceptibility are not fully understood, although it is likely to play a role the reduced brain activity of MAO-B (with a consequent reduction of MPTP biotransformed into the active metabolite MPP +) (Inoue et al., 1999; Staal et al., 2000).

- Rotenone

An experimental model of Parkinson's disease in rats recently introduced is based on chronic administration of rotenone (Betarbet et al., 2000), an organic insecticide used in agriculture, known for being one of the most potent inhibitors of mitochondrial complex I. The rationale for its use as a "parkinsonian" neurotoxin comes from the notion that nigral neurons of patients are burdened by a deficiency of complex I and the potential role that prolonged exposure to certain environmental toxins would have in the onset of the disease (Petrovitch et al., 2002).

Systemic administration of rotenone causes a selective degeneration of nigrostriatal neurons and resulting dopaminergic denervation of the striatum.

Above all, and this is the element that distinguishes the model of rotenone from others, it determines the appearance of cytoplasmic inclusions in nigral neurons, similar to typical inclusions of Parkinson's disease (the already mentioned Lewy's bodies) for the presence of α -synuclein, ubiquitin and for ultra-structural characteristics (Betarbet et al., 2000).

Subsequent studies carried out in vitro have demonstrated that prolonged exposure to rotenone causes an increase in intracellular α -synuclein, initially of the soluble fraction and further the insoluble, thus creating a condition for the formation of protein precipitates (Sherer et al., 2002).

Recent data on mechanisms of neurotoxicity rotenone demonstrate that cell death is directly related to the pro-oxidant effect, rather than the energy deficit (in terms of reduced synthesis of ATP) caused by the toxin (Sherer et al., 2003).

The criticisms of this model mainly concern the lack of selectivity of toxic action, which some authors may also extend to other basal ganglia and brainstem structures, such as the locus coeruleus and nucleus-pontine peduncle (Höglinger et al., 2003).

7.2. Transgenic models

A further development of the modeling of Parkinson's disease has been the introduction of transgenic models, which are based on the induction ("knock-in") of the expression of the gene coding for the human α -synuclein, both in normal form

("wild type") and in forms bearing one or both mutations linked to familial Parkinson (A53T and A30P).

The models used are basically two types and they differ depending on whether the expression of the gene for the α -synuclein is ubiquitous, or restricted to the nervous system (Kirik and Bjorklund, 2003). In the first case, the transgene expression is induced systemically, using plasmids as a vector during the early stages of embryonic development of the animal (mouse). The overexpression of α -synuclein, especially in the mutated form, induces intraneuronal accumulation of the protein, with the formation of cytoplasmic inclusions in various brain areas (cortex, hippocampus, substantia nigra) and motor deficits, not necessarily associated with marked deficit of the nigrostriatal functionality.

In fact, using this model, alterations at the level of striatal dopaminergic terminals have been described without, however, the detection of Lewy's bodies inclusions at the level of the substantia nigra (Giasson et al., 2002; Masliah et al., 2000; van der Putten et al., 2000).

This technique has been further refined using a promoter of the gene for tyrosine-hydroxylase, in order to promote the preferential expression of the gene for the α -synuclein in dopaminergic nigrostriatal neurons. This has in fact resulted in a greater specificity of alterations, with a selective deposition of α -synuclein in the substantia nigra and the development of motor abnormalities associated with reduced levels of striatal dopamine in older animals (Matsuoka et al., 2001; Richfield et al., 2002). However, even in this case nigral neurodegenerative events have not been observed.

In the second model of transgenic PD, α -synuclein expression is induced selectively in the substantia nigra of adult mice by direct infusion of adeno-viral vectors bearing, in their single-helix of DNA, the gene of human α -synuclein, normal or mutated. In this case, the alterations are more evident, with a clear degeneration in nigral neurons, the presence of cytoplasmic inclusions of Lewy's bodies positive for α -synuclein, reduction of striatal dopamine and motor abnormalities (Kirik and Bjorklund, 2003). The fact that the pathological and functional changes are more marked is most likely due to the anatomical selectivity and greater amount of transgenic expression of α -synuclein achievable with this technique.

The data obtained so far with both models do not solve, however, a fundamental question: it is not known yet if the accumulation of α -synuclein in the substantia nigra is the cause or effect of the neurodegenerative process in Parkinson's disease.

Research's Aim

Aim 1: To investigate the consequences of an acute nigrostriatal damage on the peripheral non-motor functions in rats, especially at the level of the digestive tract.

The main symptoms of Parkinson's disease consist in motor and extra motor disorders, the latter ones, affecting the gastrointestinal (GI) system, were and remain today object of interest with the aim to understand their genesis and to identify the best therapy.

Their early manifestation compared to the onset of motor disorders has led some researchers to speculate that the origin of the neurological damage is detectable exactly in the gut and in particular in the enteric nervous system, from which it would spread to the superior nerve centres (Braak et al., 2003). To provide scientific evidence that allows to know the origin of the extra motor disorders to support or complete these considerations, this research aims at evaluating, in a classic experimental model of Parkinson's disease, whether a damage of nigrostriatal dopaminergic pathways can account for peripheral function abnormalities, particularly gastrointestinal dysfunctions, and aims at investigating the molecular mechanisms involved. For this purpose, Sprague Dawley rats underwent intracerebral microinjection of 6-OHDA to cause a central dopaminergic deficit, they were monitored in the next eight- nine weeks to follow the evolution of changes in gastrointestinal transit, growth and behaviour doing mostly in vivo experiments.

The development of gastrointestinal motility disorders was studied in vivo monitoring weekly the 24 hours fecal output and every 4 weeks from central lesion the orofecal transit in rats after gavage of unabsorbable marker. Regional motor derangements were evaluated in the same frame time by radiographic analysis of gastrointestinal tract after a radiopaque bolus administration and through the determination of Geometric Centre. Colonic transit was also examined measuring beads expulsion time.

Regarding the in vitro experiments, the motor response of isolated pyloric, circular and longitudinal strips of stomach to contractile or relaxant agents, and to electrical field stimulation has been analysed after 8 weeks by surgery. Particular attention has been paid to the study of responses to dopamine, the neurotransmitter also present in

SNE with inhibitory role for which some authors have identified variations in turnover and receptor availability especially in the GI of experimental models of Parkinson's disease. Since some experimental observations of immunohistochemistry, obtained by other researchers (Colucci et al., 2012) on the same model, had suggested an alteration in the nitrergic neurotransmission, the tissue responsiveness has been studied even after inhibition of the enzyme NO synthase. Finally, to verify the hypothesis that there is a relationship between gastrointestinal motility disorders that accompany the early stages of Parkinson's disease and impaired vagal transmission (Pfeiffer, 2011), it has been evaluated the motor response of the pylorus and gastric circular and longitudinal strips to the transmural electrical stimulation, able to cause release of endogenous acetylcholine and norepinephrine - as well as other enteric neurotransmitters.

Since the intracerebral administration of 6-OHDA causes selective destruction of dopaminergic cells by generation of oxygen free radicals (Baluchnejadmojarad, 2010) and patients with Parkinson's disease have high intracerebral and spinal fluid levels of ROS (LeWitt et al ., 2011), it was investigated whether a condition of oxidative stress could also occur at the peripheral level. Thus the content of malondialdehyde has been determined, an index of lipid peroxidation, in different portions of the gastrointestinal tract, bladder and kidney of animals lesioned with 6-OHDA; the results were compared with those obtained in control animals.

Aim 2: To evaluate the effects of oral subchronic treatment with L-DOPA / Benserazide in the PD model

The first choice treatment of Parkinson's disease is still based on the use of L-DOPA, the physiological precursor of dopamine, associated with an inhibitor of peripheral L-amino acid decarboxylase like Benserazide, which facilitates L-DOPA access to the CNS and reduces its peripheral side effects. Indeed L-DOPA treatment could cause gastrointestinal disorders such as gastroparesis or delayed gastric emptying, abdominal pain, constipation, diarrhea, nausea and vomiting that worsen the digestive tract dysfunctions typically present in PD patients. Furthermore PD patients' altered GI activity can affect L-DOPA pharmacokinetics impairing the outcomes of the therapy. In addition, long-term treatment with L-DOPA has been associated with the

development of abnormal involuntary movements known with the term dyskinesia (Bordet et al., 1997).

Accordingly, in this second part of the thesis, the peripheral effects on the gastrointestinal tract (and at the level of other regions) of an oral sub-chronic treatment with L-DOPA/Benserazide have been evaluated in the experimental model of Parkinson's disease in rats. The consequences of 5 weeks oral administration of L-DOPA/Benserazide (6 mg/kg and 15 mg/kg respectively) on gastrointestinal tract motility, metabolic parameters and animal behaviour were evaluated in rats submitted to 6-OHDA intracranial injection 4 weeks before the pharmacological treatment.

In particular, to assess the total gastrointestinal functionality in vivo, functional investigations have been performed including determination of 24 hours fecal output and orofecal transit. The evaluation of regional dysmotility was performed in a quantitative way by estimating the Geometric Centre, by radiographic analysis of the digestive tract after a radiopaque bolus administration and by measurement of colonic transit. Finally, to obtain information regarding the effects induced by drug treatment on the degree of oxidative stress, the tissue levels of malondialdehyde (MDA), a marker of lipid peroxidation, was quantified.

Aim 3:

- A.** To investigate the rhythmic pan-colonic propulsive motor patterns and their spatiotemporal organization in control and PD rats.
- B.** To ascertain role and involvement of ICC (Interstitial Cells of Cajal) in gastrointestinal motor dysfunctions associated with the central dopaminergic damage in the rat model of PD.

To go deeper into the cellular mechanisms involved in the pathogenesis of the dysmotility affecting GI system, it became crucial to identify which neurotransmitters, neuronal networks or motility patterns could be compromised in association with central dopaminergic deficit. Our attention was addressed to ICCs, fundamental pacemaker cells responsible for the correct coordination of GI motility. These cells are particularly sensitive to oxidative stress and their deregulation is often involved in different GI disorders, from inflammation to chronic constipation (Huizinga et al., 2011). For this reason it has been planned a collaboration with the qualified research

centre in McMaster University (Canada) directed by Dr Jan Huizinga an excellent pharmacologist, internationally recognized for his high level competence in the study of the motility of the gastrointestinal tract and his extensive experience with immunohistochemistry of Interstitial Cells of Cajal (ICC).

It has been possible to study the ICCs, to identify and recognize their different types through the whole GI tract, to visualize and quantify them in control conditions and in the experimental model of PD induced in rats.

In parallel with the immunohistological investigation on stomach and colon segments excised from control and PD rats, it has been possible to study the spontaneous and pharmacologically stimulated motility patterns of colon isolated from control and PD rats, to monitor and quantitatively describe the rhythmic pan-colonic propulsive motor patterns. Indeed, this laboratory has as well a huge experience with the investigation of intestine motor patterns and of their control mechanisms through video recording and subsequent spatiotemporal mapping of the whole colon in vitro, a technique developed and reproduced exclusively there.

The study of the ICC presence and the propulsive colonic motor patterns on Sprague-Dawley rats bearing nigrostriatal lesions caused by 6-hydroxydopamine (6-OHDA), which reproduces the central dopaminergic denervation typical of PD and develop severe constipation (Blandini et al., 2009), was performed also thanks to the collaboration with a research group expert in Parkinson's disease headed by Dr Len Niles that reproduced this specific PD model in rats.

Materials and Methods

The research has been conducted in compliance with the current European regulations (*European Communities Council Directive 86/609/EEC of 24 November 1986*) and Italian law (*D.Lgs 116/1992 and D.Lgs 26/2014*), on the protection and use of laboratory animals. Male Sprague-Dawley rats (Charles River Laboratories, Calco, CO, Italy) were used for all the experiments, weighing between 250 and 280 g. The animals were housed at a temperature of 23 +/-1 °C and a relative humidity of 50% on a 12h dark-light cycle with water and food ad libitum. Before starting the experiments they were divided randomly into two groups: control and 6-OHDA animals.

6-hydroxydopamine (6-OHDA), dopamine, prostaglandin F₂ α (PGF₂ α), bethanechol, sulpiride, suramin, atropine, guanethidine, α -chymotrypsin and N ω -Nitro-L-arginine methyl ester (L-NAME) derive from Sigma-Aldrich Co. LLC. (St. Louis, Missouri, United States), KCl and NaCl were purchased from Merck (KGaA, Darmstadt, Germany) while L-ascorbic acid is from the company Carlo Erba Reagents (Milan, Italy). The dyes Evans Blue and Carmine Red and 3, 4-Dihydroxy-L-phenylalanine (L-DOPA) and Benserazide hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Surgical procedure

The intracerebral administration of 6-OHDA is carried out in anesthetized animals (sodium thiopental 50 mg/kg intraperitoneal) by stereotaxic procedure (Fig.14).

After placement of the animal in the stereotaxic apparatus and the subcutaneous injection of lidocaine 2%, the skull is exposed and Bregma is identified as reference point for the coordinates that identify two sites of intervention at the level of the nigrostriatal bundle (MFB medial forebrain bundle) (Blandini et al., 2009) (Fig.15).

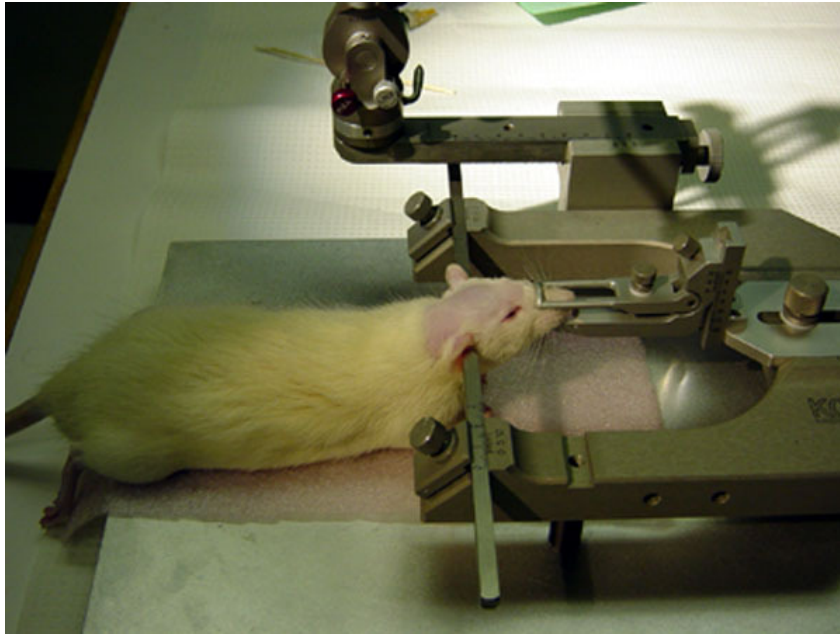


Fig. 14 - Rat positioned on the stereotaxic device
(Taken from A Photographic Series, Cooley et al., 1990)

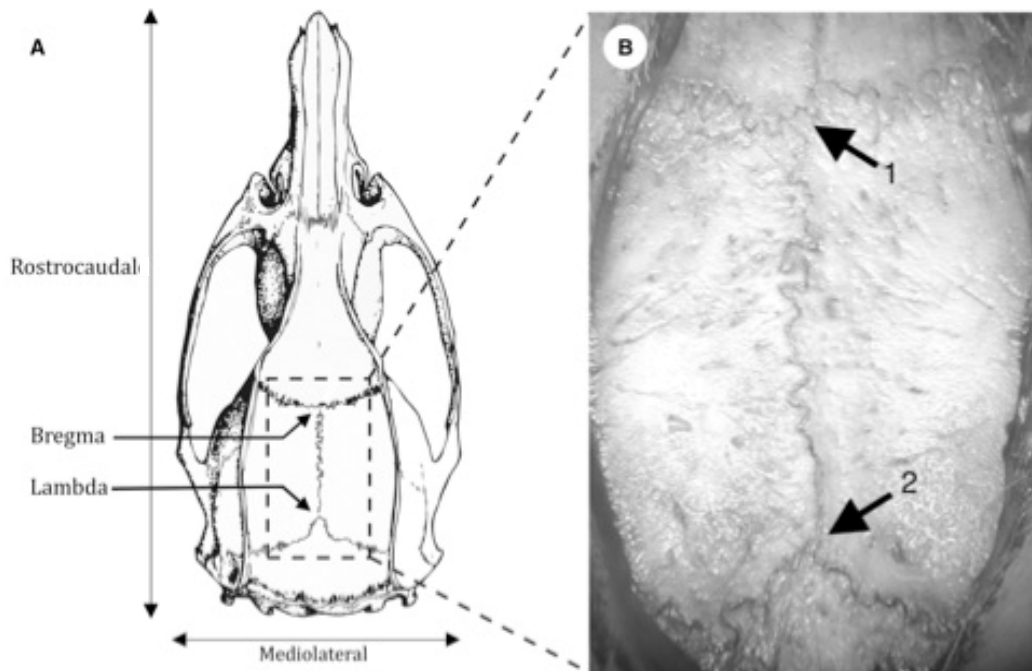


Fig. 15 - Sutures of the skull (A and B). Between point 1 and point 2 shall there is the MFB (Medial Forebrain Bundle). Redeveloped from Ruitenberg et al., 2002 (www.sciencedirect.com):

1: anteroposterior (AP) -4.0; mediolateral (ML) -0.8; ventrodorsal (VD) -8.0, with the teeth fixing bar to 3.4; INJECTION 3 μ l of 6-OHDA, 3 minutes.

2nd: anteroposterior (AP) -4.4; mediolateral (ML) -1.2; ventrodorsal (VD) -7.8, with the tie bar teeth to -2.4; INJECTION 2.5 μ l of 6-OHDA, in 2.5 minutes.

The animals of 6-OHDA group underwent to microinjection of 6-OHDA in the two sites of the nigrostriatal pathway, similarly the animals of control group were treated with comparable volume of saline. From Bregma, which is the point of convergence between the coronal suture and sagittal skull, coordinates are calculated to make two injections:

- **1st injection:** anteroposterior (AP) -4.0; mediolateral (ML) -0.8; ventrodorsal (VD) -8.0, if the fixing bar of the teeth is 3.4.
- **2nd injection:** anteroposterior (AP) -4.4; mediolateral (ML) -1.2; ventrodorsal (VD) -7.8, if the fixing bar of the teeth is at -2.4

The neurotoxin 6-OHDA (solution in 0.05% ascorbic acid) is injected using a 10 μ l Hamilton microsyringe, at the speed of 1 μ l per minute, in a volume of 3 μ l for the first injection and 2.5 μ l for the second. The needle is left in place for four minutes before the retraction to facilitate the circulation of the solution and to prevent backflow. The neurotoxin is administered only in the right hemisphere, while the untreated contralateral region is used as an internal control. At the end of the surgical operation, the tissue flaps are sutured using metal staples.

Rotational behavioral test

Apomorphine is a dopaminergic D2 agonist used to verify and quantify the entity of nigrostriatal lesion after two weeks form surgery. Apomorphine, dissolved in 0.02% ascorbic acid, is administered subcutaneously at a dose of 0.1 mg/kg to animals (control and lesioned groups) that are monitored in the following 40 min. In lesioned rats, apomorphine produces complete rotations in controlateral direction relative to the lesioned hemisphere and the frequency of rotations, which does not occur in control rats, is an index of the degree of lesion produced (Przedborski et al., 1995).

The chosen dose allows us to highlight lesions with more than 50% of entity and the rats presenting a dopaminergic deficit greater than 90% perform a number of rotations higher than 125/30' (Truong et al., 2006). The number of animals subjected to 6-OHDA injection and entering the experimentation were 44 whereas the control were 40.

Measurement of physiological parameters

After a week of post-operative stabilization, the rats are weekly monitored measuring their weight. Once a week and for a cycle of eight-nine weeks they are placed in metabolic cages for 24 hours in order to measure the faecal output (g/24h), food intake (g/24h), urine production (ml/24h) and water consumption (ml/24h).

Gastrointestinal (GI) transit is evaluated after 4, 6 and 8 weeks from surgery in terms of **oro-fecal transit**, **colonic transit** and through **radiographic analysis**. At the conclusion of the experiment (after 8-9 weeks) the GI motility is evaluated by the determination of **the Geometric Centre** through the administration of a non-absorbable dye and portions of the digestive tract are analyzed by measuring the concentration of the marker of lipid peroxidation, malondialdehyde (MDA).

Treatment with L-DOPA/Benserazide

Starting from the fifth week after the surgical operation the rats belonging to each group (control and 6-OHDA) are re-randomized and divided into two more groups: treated and untreated.

The dose of L-DOPA/benserazide (respectively 6 mg/kg and 15 mg/kg) is dissolved in 1.5 ml of distilled water and administered intragastrically (gavage) once daily for the duration of 4 weeks.

The untreated group is administered, in the same manner, an equal volume of water alone.

Oro-fecal transit

The transit through the whole gastrointestinal tract can be measured as the time taken by a non-absorbable gelatin solution of Evans Blue dye to transit throughout the GI tract and be expelled after intra-gastric administration. The solution of Evans Blue, dissolved in 0.5% methylcellulose, is administered by gavage to rats fasted for 18

hours. The animals are placed in their cages, with food ad libitum. The oro-fecal transit is calculated as the time (min) that elapses between administration of the marker and the expulsion of the first (t_1) and the last (t_2) marked pellets. This analysis is complementary to the transit measured by the method of geometric center because, even if it does not offer information about the contribution to motility of the gut single portion, it might be repeated more times in the same animals during the course of eight-nine weeks after surgery.

Colonic transit

Colonic transit is evaluated following the method of Raffa et al. (1987). According to this method, the time required for the expulsion of a 5mm diameter glass bead inserted 3 cm backward into the distal colon is measured, and it is considered as colonic expulsion time.

After 4, 6 and 8 weeks from the induction of the central lesion, overnight fasted rats were housed in single cages to provide a measure of colonic propulsion plus expulsion of beads past the internal and external anal sphincters. The time for expulsion of the bead was recorded and expressed in min.

Gastrointestinal transit – Geometric Centre method

The total GI transit is measured according to the method of De Winter (1997).

Overnight fasted rats received intragastrically 1 ml Evans Blue, a semiliquid non-nutrient dye (50 mg/ml dissolved in 0.5% methylcellulose). After 1 h rats were euthanized by CO₂ inhalation and the whole GI tract was carefully removed. The stomach was numbered as segment 1, the small intestine was divided into 10 equal segments (numbered as 2–11), the caecum was numbered as 12 and the large intestine was divided into 3 segments (13–15). Each segment was placed in 25 ml 0.1 M NaOH and minced and then stirred for 30 sec. The resulting suspension was left at room temperature for 1 h. The supernatant (5 ml) was then centrifuged at 1356 g for 20 min. Samples were further diluted (1:5 for intestinal specimens and 1:50 for the stomach), and absorbance (Abs) was read at 550 nm (De Schepper et al., 2007). The results were expressed as geometric center (GC), according to the formula: $GC = \frac{\sum (\% \text{ Abs Segment} \times N \text{ Segment})}{100}$.

At lower values of GC corresponds a decline in the speed of transit through the digestive tract. It is also possible to evaluate the distribution of the dye along the

gastrointestinal tract to obtain the contribution of the individual segments to the total motility (Fig.16).

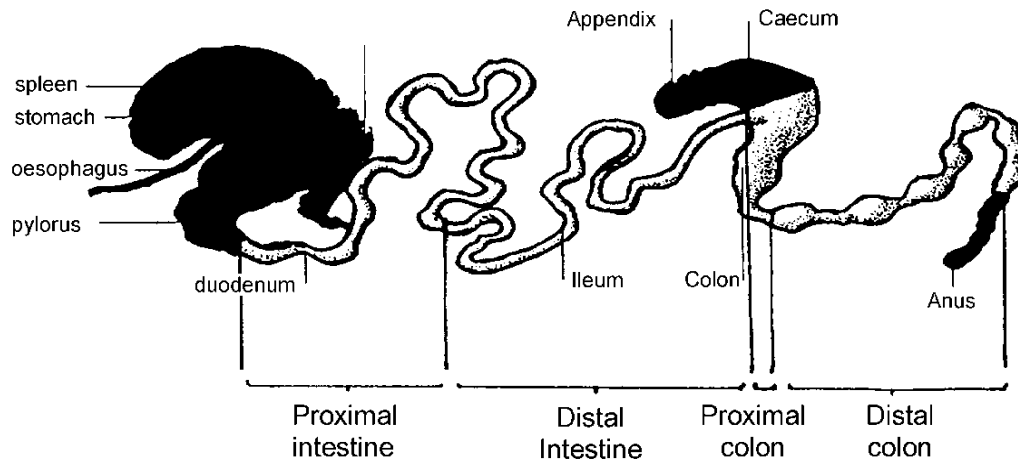


Fig. 16a Gastrointestinal tract layout.



Fig. 16b - Fifteen segments form GI tract placed in 25 ml 0.1 M NaOH and minced.

Radiological analysis

After 4, 6 and 8 weeks from the induction of the central lesion, overnight fasted rats received a suspension of BaSO₄ (2.5 ml, 1.5 g/ml) (Prontobarrio H.D. Bracco Imaging Italia, Milan, Italy) intragastrically and radiographic exposures were performed at 0, 0.5, 1, 2, 10, 11 and 12 h after barium sulphate administration. Focus-film distance was manually fixed at 100 cm and exposure values were 65 kVp–4.5 mAs (exposure time: 0.01 sec). Total body dorso-ventral radiographic projections were considered. Rats were housed in restrainers (plexiglass tube-shaped cages closed at the extremities), adjusted to the size of the animals, so that they could easily accommodate and not move, escape or turn around, to avoid the use of any anaesthesia. The analysis of radiographic images was carried out according to the scoring proposed by Cabezos et al. (2008) by 6 different observers blinded to the treatment. In particular, the proportion of the organ labelled, the intensity of labelling, the organ profile, and the homogeneity of labelling in the organ were evaluated in four GI regions (stomach, small intestine, caecum, colorectal region) and scored, to obtain an overall value ranging from 0 to 12.

Evaluation of gastrointestinal motility

	Proportion of the organ labelled	Intensity of labelling	Profile of the organ*	Homogeneity of labelling in the organ*
0	No labeling			
0–1	<25%	Faint (shadow)	Not well-defined	Heterogeneous
2	25–50%	Light	Well-defined	Homogeneous
3	50–75%	Moderate	–	–
4	75–100%	Strong	–	–

Four gastrointestinal regions were evaluated: stomach, small intestine, caecum, colorectal region (faecal pellets). Each region was scored considering four parameters, to give a final compounded value from 0 to 12. Evaluation focused on the more intensely labelled area in each region. As an exemption, stomach and caecum were considered as a whole when profile and homogeneity (*) were evaluated.

Fig. 17 Evaluation of gastrointestinal motility.

Cabezos et al., 2008

In vitro functional studies on stomach and pylorus

- Withdrawal samples of tissues and organs

After eight weeks from stereotaxic surgery, control and 6-OHDA rats are sacrificed by carbon dioxide inhalation.

Following laparotomy, organs of the abdominal cavity are exposed, the stomach is quickly removed and dissected for the preparation of isolated tissues: pylorus, strip of circular smooth muscle of the gastric fundus and longitudinal smooth muscle strips obtained from the great curvature of the stomach and including Fundus, Corpus and Antrum (Fig. 18,19)



Fig. 18 – Abdominal Anatomy of a male rat

1 Liver 2. Caecum 3. Ileum 4. Testis 5. Ectoderma and mesoderm 6. Stomach 7.
Greater Omentum 8. Spleen

(M. Perkins, Orange Coast College)

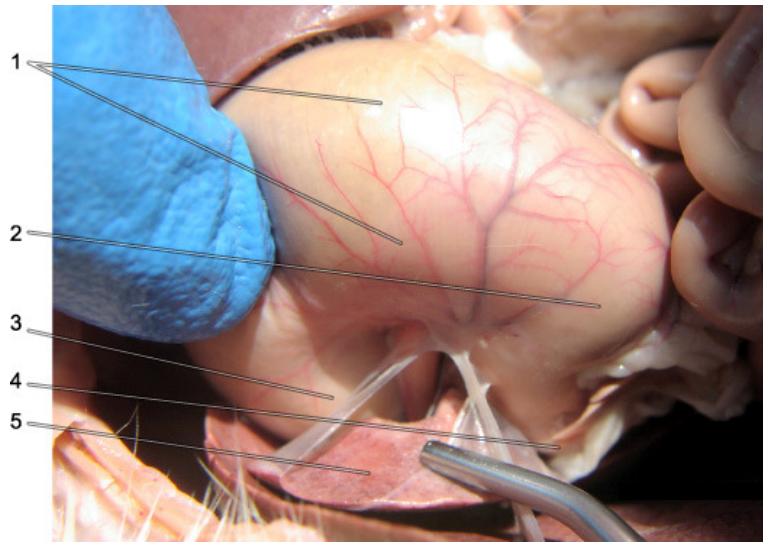


Fig. 19 - Anatomy of the stomach of a male rat

1. Body 2. Antrum 3. Fund 4. Pyloric sphincter 5. Liver

(M. Perkins, Orange Coast College)

➤ Preparation of gastric strips in organ baths

Segments of different tissues, long about 2 centimeter, were mounted longitudinally in baths for isolated organs (Fig. 20, 21, 22), each containing an appropriate nutrient solution:

Modified H. Krebs solution, 2000 ml (for Circular gastric Fundus Strip) (Shichijo et al., 1997)

NaCl	13.86 g
KCl	0.72 g
CaCl ₂ X 2H ₂ O	0.56 g
KH ₂ PO ₄	0.32 g
MgSO ₄ X 7H ₂ O	0.60 g
NaHCO ₃	4.20 g
Glucose	3.64 g

Krebs-Ringer solution, 2000 ml (for Pylorus) (Puri et al., 2002)

NaCl	15.54 g
KCl	0.70 g
CaCl ₂ X 2H ₂	0.74 g
NaH ₂ PO ₄	0.38 g
MgSO ₄ X 7H ₂ O	0.30 g
NaHCO ₃	2.76 g
Glucose	2.78 g

Tyrode solution, 2150 ml (for Longitudinal Gastric Strip) (He et al., 2006)

NaCl	17.2 g
KCl	0.43 g
CaCl ₂ X 2H ₂ O	0.43 g
MgCl ₂ X 6H ₂ O	0.215 g
NaH ₂ PO ₄	0.107 g
NaHCO ₃	2.15 g
Glucose	2.15 g

The nutritive solutions, thermostated at 37 ° C, were oxygenated with Oxico gas (a mixture of 95% O₂ and 5% CO₂) and the tissues were subjected to a resting tension of 1 g.

After a stabilization period of about 30-45 minutes (during this time the solution was renewed at regular intervals of 15 minutes), the tissues were stimulated pharmacologically or electrically: motor responses were recorded by means of an isometric transducer, connected to a pen recorder (model Gemini 7070, Basile, Comerio, Varese, Italy).

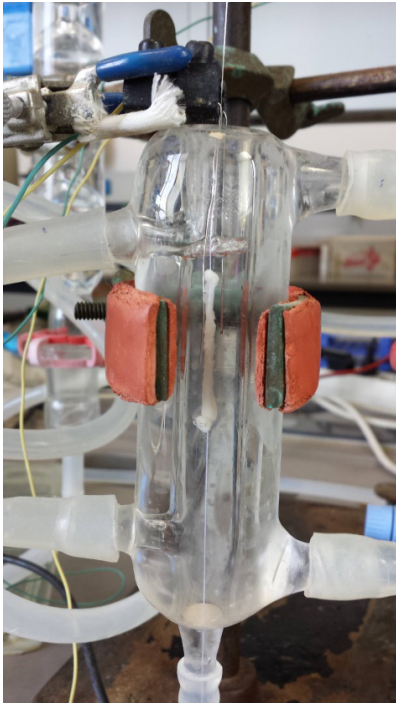


Fig. 20 - Circular gastric Fundus Strip

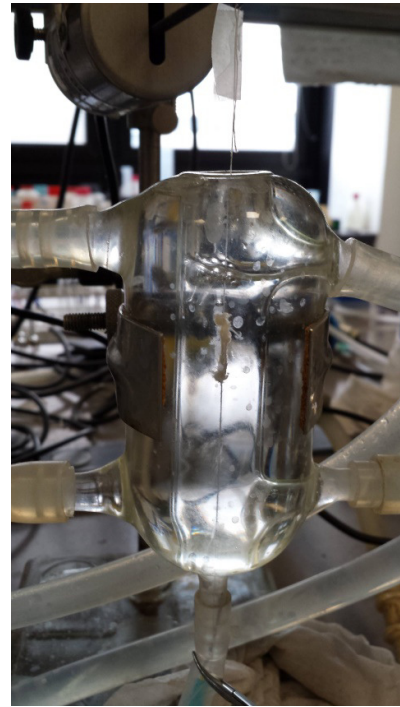


Fig. 21 - Pylorus

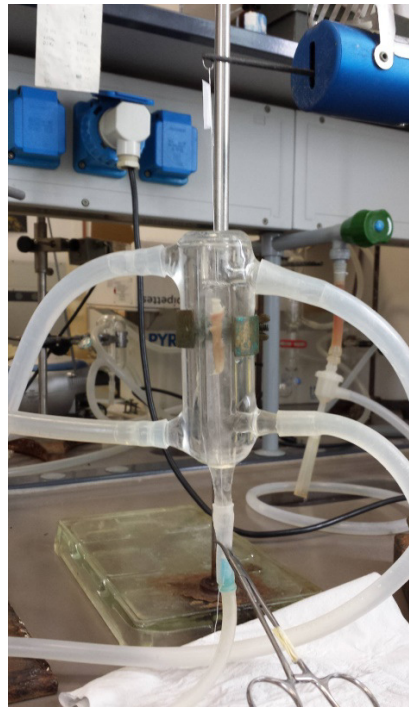


Fig. 22 - Longitudinal Gastric Strip

➤ Experimental protocols

Circular and Longitudinal Gastric Strips

- Pharmacological Stimulation

Both longitudinal and circular strips were initially contracted by the application of 30 mM KCl. After restoring the basal tone, increasing cumulative concentrations of bethanechol (10^{-7} M - 10^{-4} M) and PGF2 α (10^{-9} M - 10^{-5} M) were applied in order to make complete concentration-response curves.

On the tissues pre-contracted with application of the EC₅₀ of PGF2 α (around 10^{-7} M), were cumulatively applied increasing concentrations of dopamine (10^{-7} M - 3×10^{-3} M) in the absence and then in the presence of 10^{-6} M sulpiride, so as to exclude the contribution of dopamine D2 and D3. After exposure to each pharmacological agent, the tissues were washed out with their respective nutrient solution and left to rest for at least twenty minutes before the next stimulation; sulpiride was incubated for ten minutes.

- Electrical Stimulation

The circular and longitudinal gastric strips were positioned between a pair of platinum electrodes connected to an electrical stimulator (LACE Electronics Mod. ES-3, PI Ospedaletto - Italy) and to a unit of isolation (Multiplex Pulse Booster Mod 3165, Ugo Basile). The tissues pre-contract with PGF2 α (10^{-7} - 3×10^{-7} M) were electrically stimulated with pulse trains lasting ten seconds at 1 minute intervals. The pulse frequency, with intensity of 120 mA and duration of 0.5 msec, has been progressively increased from 1 to 10 Hz. This procedure allowed us to build up frequency-response curves in basal conditions and in NANC conditions (not adrenergic, not cholinergic), produced by applying the nutrient solution medicated with atropine 4×10^{-6} M and guanethidine 10^{-6} M. In order to exclude a relaxation mediated by release of nitric oxide or by peptidergic transmission, L-NAME, NO synthase inhibitor (3×10^{-4} M), and protease α -chymotrypsin (40 U/ml) were added later in sequence.

Pylorus

- Pharmacological Stimulation

On the strip of the pylorus, longitudinally mounted, we evaluated the contractile effects generated by the administration of 30 mM KCl and the application of cumulative increasing concentrations of bethanechol (10^{-7} M - 10^{-3} M). On hypertone generated by bethanechol (concentration which gives 50% of the maximum effect, between 10^{-5} and 10^{-4} M) increasing concentrations of dopamine (10^{-7} M - 3×10^{-3} M) were applied in the absence and in the presence of 10^{-6} M sulpiride left to incubate for 10 minutes before agonist administration.

- Electrical Stimulation

The electrical stimulation was performed in the same conditions described above, on tissues pre-contracted with bethanechol (concentration which gives 50% of maximum effect, between 10^{-5} and 10^{-4} M). Frequency-response curves were constructed in the absence and in the presence of L-NAME (3×10^{-4} M) or suramin (10^{-4} M), P2 purinergic receptor antagonist.

Also in this case, after the electrical stimulation, two concentration-response curves of dopamine (10^{-7} M - 3×10^{-3} M) were made in the absence and in the presence of sulpiride 10^{-6} M. The curve concentration-response of bethanechol (10^{-7} M - 10^{-3} M) has been also repeated, always in order to detect a possible change of the tissue responses as a result of electrical stimulation.

At the end of each experiment, all the tissues were dried with filter paper and weighed.

➤ Data analysis

The responses (tension g) produced by the different tissues have been normalized in relation to the weight of the tissue and expressed as g per weight of wet tissue (g/g tissue). This procedure allowed the comparison of motor responses produced by different tissues, both in terms of sensitivity to various pharmacological agents tested and the transmural electrical stimulation both in terms of contractile efficiency of the muscular component.

The potency of the different agonists tested was expressed as pD₂ (negative logarithm of EC₅₀, concentration able to produce 50% of maximal response), calculated by the linear regression analysis applied to the concentration-response curves of each agonist.

In vitro functional study of colonic motility

➤ Preparation of the colon

All procedures were performed in accordance with the regulations issued by the Animal Ethics Board of McMaster University.

After removal of the colon from abdomen, the luminal content is completely wash out with warm Krebs solution (composition for the preparation of 1.5 L: NaCl 10.55 g, KCl 0.660 g, MgCl₂.6H₂O 0.366 g, CaCl₂.2H₂O 0.551 g, NaH₂PO₄.H₂O 0.248 g, NaHCO₃ 4.2 g, glucose 4 g, pH 7.4). The colon is fixed with pins in a dish for dissection containing fresh and oxygenated (95% O₂ and 5% CO₂) Krebs solution and it is cleaned from the portion of mesenteric fat under optical microscope and using fine-tipped scissors.

➤ The organ bath and the multicamera system

The bath apparatus for isolated organs consists of several parts essential to keep the tissue or organ alive, in order to carry out experiments and data recording. The main bath has rectangular shape and it is filled with 1.5 liter of Krebs solution. It contains a

mobile plastic bar ("kit kat") that is divided into 3-5 compartments or separate lanes for the arrangement of the colon (Fig. 23). All around are prepared:

- 1) a large diameter pipe with circulating warm water connected to a pump/heater of water maintaining the Krebs solution at a temperature of 37°C,
- 2) a tube with narrow diameter (with holes applied using a needle) containing a mixture of 95% O₂ and 5% CO₂. From one side of the bath there is a reservoir system of saline solution (NaCl 3.945 g, KCl 0.186 g in 500 ml) mounted on an adjustable rod; in this way, raising or lowering the reservoir, the intraluminal pressure in the colon can be adjusted or changed. The reservoir consists of 50 ml syringes with valve connected by tubing with the proximal end of the colon. The distal end is connected with other tubes that allow the outflow of the saline solution and the luminal contents (mucosa etc.) in separate containers. The temperature of Krebs solution is monitored by placing a sensor in contact with the solution in the bath, the signal is recorded in a computer using AxoScope 10.3 software. Two transparent plastic bars are laid against the two long sides of the bath necessary to prevent the diffusion of the bubbles along the surface of the solution since it would interfere with the recording of the experiment. There are two other tubes that connect the bath with two air pumps (AW-20 aqualifter, Tom Aquarium Products, Shawnee, KS) that let to circulate the Krebs solution, essential for maintaining a long-term viability of the colon. Finally three sources of led light provide the right lighting to the bath in its length.

Above the bath there are mini digital video cameras (n = 10, 1/3 'SONY Super HAD CCD, 700 TVL, SONY Effio-E DSP with lens focal length 50 mm (F2.0; AOV 9th) purchased by Security Camera 2000 (Hong Kong), which record visual contractions of the tissue. Video recordings are made simultaneously with two clicks on a computer connected to a recorder (Vonnice K4116HMF, Markham, Ontario) once established the desired intraluminal pressure.

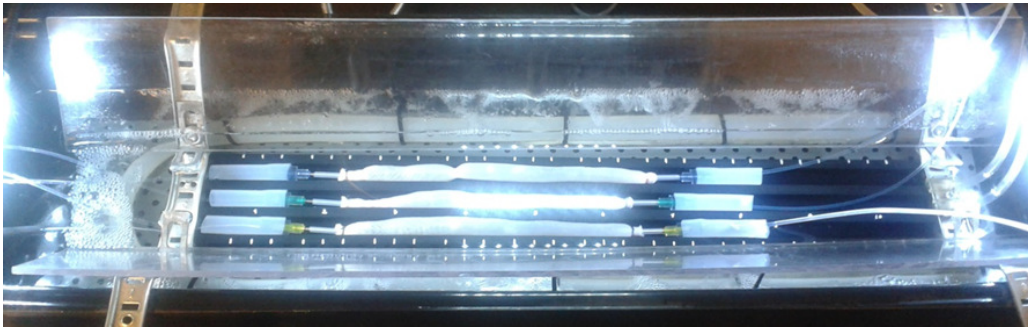
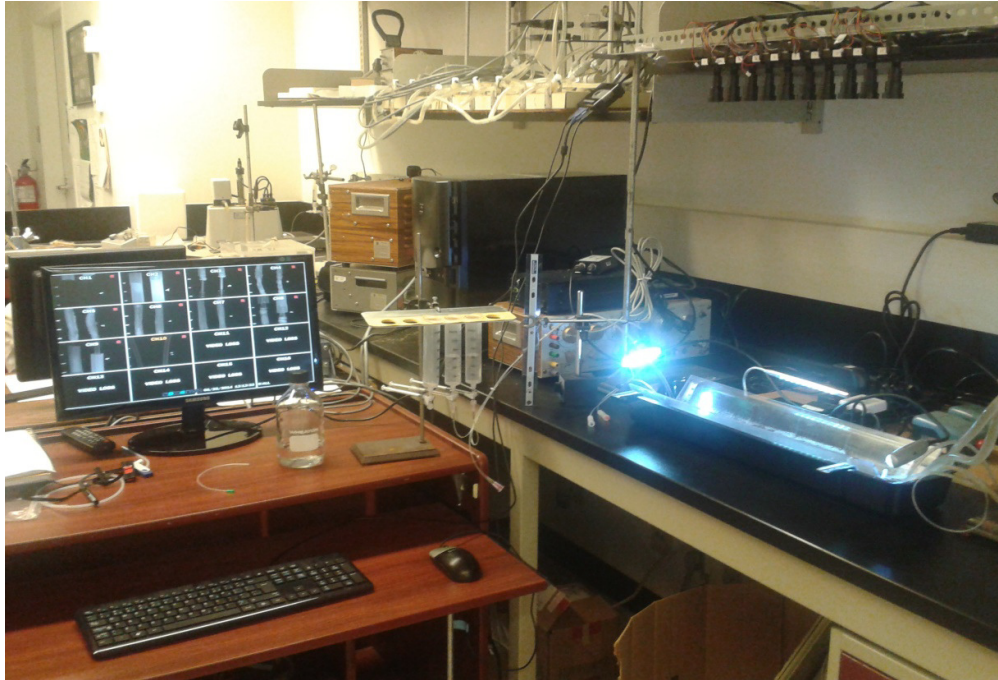


Fig. 23 - Organ bath set up for intestine motility study.

➤ Experimental protocols and production of spatiotemporal Maps

Several experimental protocols have been tried, aimed at the evaluation of motor patterns (LDC = Long Distance Contraction, RPMC= Rhythmic Propulsive Motor Complexes, PPA= Proximal Pacemaker Activity and Ripples), in normal conditions (Base Line or Stabilization 60 min) and in the presence of D2 receptor antagonist for an incubation time of 30 min. When it was considered necessary to change the solution of the bath in order to administer different drugs and see the individual effect of each of them, a pump has been mounted that helps to remove the old solution from the bath and then add a new one.

Once established the colon in the new solution and after 25 min Dopamine 3×10^{-6} M is added for 35 min. Once finished the experiment, AVI recordings are transferred from the recorder to a USB device or the computer itself, then they are transformed into MOV format videos readable by the program Quick Time, an operation that requires an hour or more. This format is essential for the next step: the conversion of video in spatiotemporal maps using the program ImagJ and specific plugins (equations) designed by Sean Parsons. Depending on the length of the video and the amount of the videos produced by an experiment, the conversion may require 24 to 48 hours. The spatiotemporal maps can be assessed in many different ways, for example in a qualitative way describing with words the aspect and shape of the motor patterns, their length, width, % of relaxation (white color) but also in a preliminary quantitative way by calculating its frequency in a definite time interval.

Immunoreactivity and quantification of c-kit positive Interstitial Cells of Cajal (ICC) and of n-NOS positive nitrergic neurons

Surgical samples of both rat stomach and colon were obtained.

They were either immersed in 4% paraformaldehyde 0.1M phosphate buffer (pH 7.4) overnight at 4°C for whole mount preparations (Fig.24, 25) or immediately embedded in OCT compound (Optimum Cutting Temperature) and frozen in liquid nitrogen. Frozen sections of ten μm were cut with cryostat (Fig. 26), mounted on coated slides and fixed with 4% paraformaldehyde for 30 min at 4°C. Both whole mount preparations and frozen sections were then washed for 30 min in phosphate buffered saline (PBS; 0.05M, pH 7.4, with 0.3% Triton X-100). Non-specific antibody binding was reduced by incubating the tissues in 2% bovine serum albumin (BSA) for 1 h at room temperature before addition of the primary antibodies. Tissues were then incubated in polyclonal goat anti c-Kit (1:200, R&D system) or polyclonal goat anti n-NOS (1:500 Abcam) for 24 h at 4°C. Secondary immunoreactions were carried out with Cy3 conjugated anti-rabbit IgG (1:400, Jackson ImmunoLab). All the antisera were diluted with 0.2% BSA in 0.3% Triton X-100 (PBS-TX, pH 7.4).

Cy3 stained frozen sections were examined with a confocal microscope (Zeiss LSM 510, Jena, Germany) (Fig. 27) with an excitation wavelength appropriate for Cy3 (592 nm) and confocal images were created with Carl-Zeiss software. All the images are

digital composites of Z-series scans of 10 optical sections through a depth of 9 micrometers. Quantification was done measuring the area of c-Kit immunoreactivity and, as the scanning depth of each preparation was the same, it was expressed as percentage of the total area or volume of the preparation. To obtain quantification data just for ICC, mast cells were recognized by their darker staining and round shape and erased from pictures before analysis. Quantification was performed using Photoshop version 7.0 (Adobe Systems, Mountain View, San Jose, CA, USA). c-Kit immunoreactivity was identified, highlighted and the area of immunoreactivity measured and expressed as percentage of the total area.

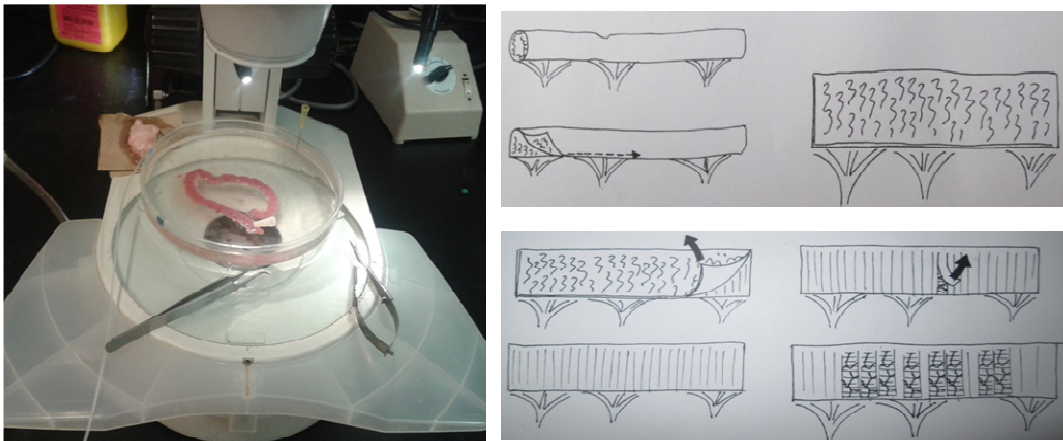


Fig. 24 - Tissue dissection and whole mount preparation.



Fig. 25 - Whole mount preparation of stomach: Fundus (white color), Corpus (reddish) and Antrum (pale red)



Fig.26 - Leica CM3050S Cryostat for cutting frozen sections.

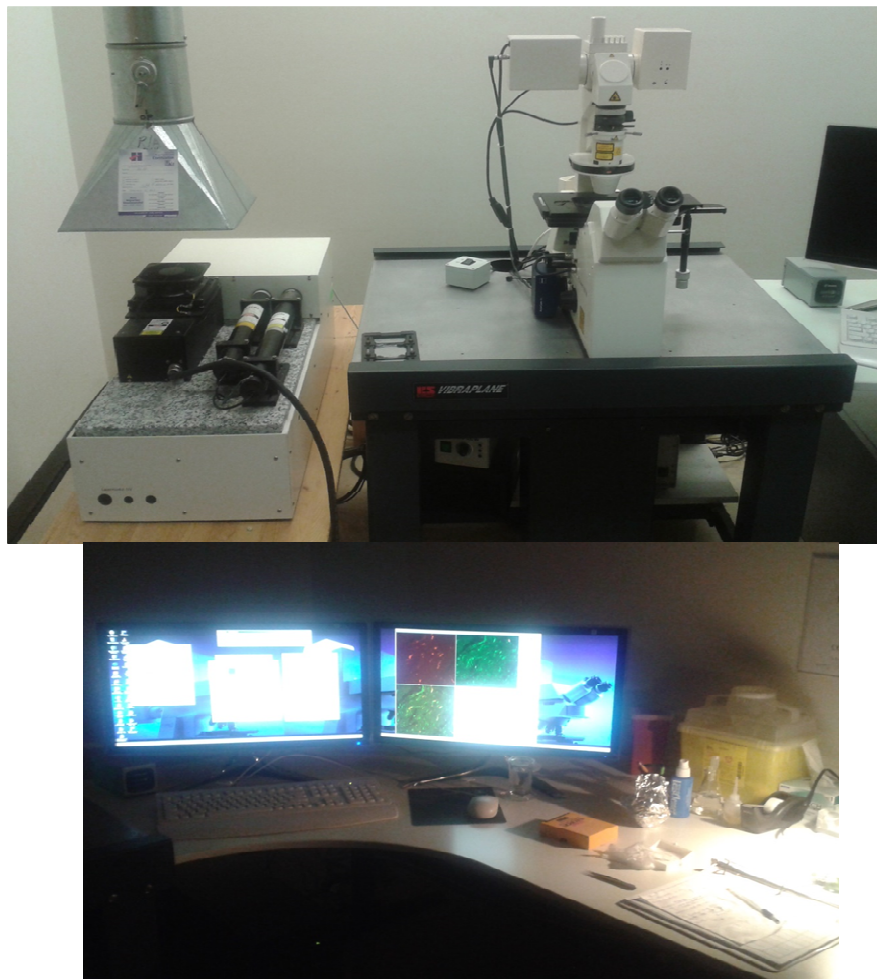


Fig. 27 - Zeiss LSM 510 Confocal Microscope.

Malondialdehyde (MDA) assay

The levels of MDA are markers of oxidative stress, in particular lipid peroxidation. The concentration of MDA is measured in different GI segments and in organs such as bladder, liver and kidney, following the method of Ohkawa (1979). Once sacrificed the animal, the various portions of the digestive tract and the organs are weighed and homogenized in 10 volumes of 1.15% KCl. An aliquot of this homogenate (300 ul) is added to the reaction solution containing 200 ul of 8.1% SDS, 1500 ul of 20% acetic acid pH 3.5, 1500 ul of 0.8% thiobarbituric acid and 500 ul of distilled water. The prepared samples were kept at 95°C for 60' to allow the reaction between MDA and thiobarbituric acid. The product of the reaction of MDA with thiobarbituric acid is extracted with butanol, in 1: 1 proportion with the volume of the reaction mixture (2ml + 2ml). After centrifugation at 3000 rpm for 10', the value of absorbance of the supernatant is read at 532 nm with a spectrophotometer and data are expressed as nmol/mg of wet tissue by referring to the calibration curve constructed with MDA (from 0.00625 to 0.2.mMol).

Statistical Analysis

The data are expressed as mean +/- standard error and analyzed statistically applying the Student's t-test for unpaired data (two tailed and one tailed) or the test of Mann Whitney, when specified, and the two-way analysis of variance (Two-way ANOVA), followed by Bonferroni post-test. The differences between groups were considered significant for values of * $p < 0.05$, ** very significant for values of $p < 0.01$ and extremely significant for values *** $p < 0.001$.

Statistical analysis was performed with the software GraphPad Prism 5.0.

Results

Part 1: Consequences of an acute nigrostriatal damage on the peripheral non-motor functions in rats, especially at the level of the digestive tract.

Behavioural test with Apomorphine

The non-selective dopaminergic agonist apomorphine, administered subcutaneously (0.1 mg/kg) after two weeks from surgery, allows to determine the entity of damage to the central dopaminergic pathways, shown by rats with transient rotational movements in contralateral direction compared to the lesioned area (Truong et al., 2006). The rotations are produced by preferential stimulation of dopaminergic receptors which, in the absence of dopamine, are subject to a hypersensitivity (Hudson et al., 1993). From the data obtained with this test, 67% of the rats lesioned with 6-OHDA has performed a number of rotations higher than 150 in forty minutes (186.8 ± 45.7), an index of damage to nigrostriatal dopaminergic fibers exceeding 90%. The remaining percentage (33%) has performed a negligible number of rotations, below the threshold value (25.2 ± 14.9) and it was therefore excluded from the experiment. The control rats, as expected, did not show any appreciable change in motor behaviour (Fig. 28).

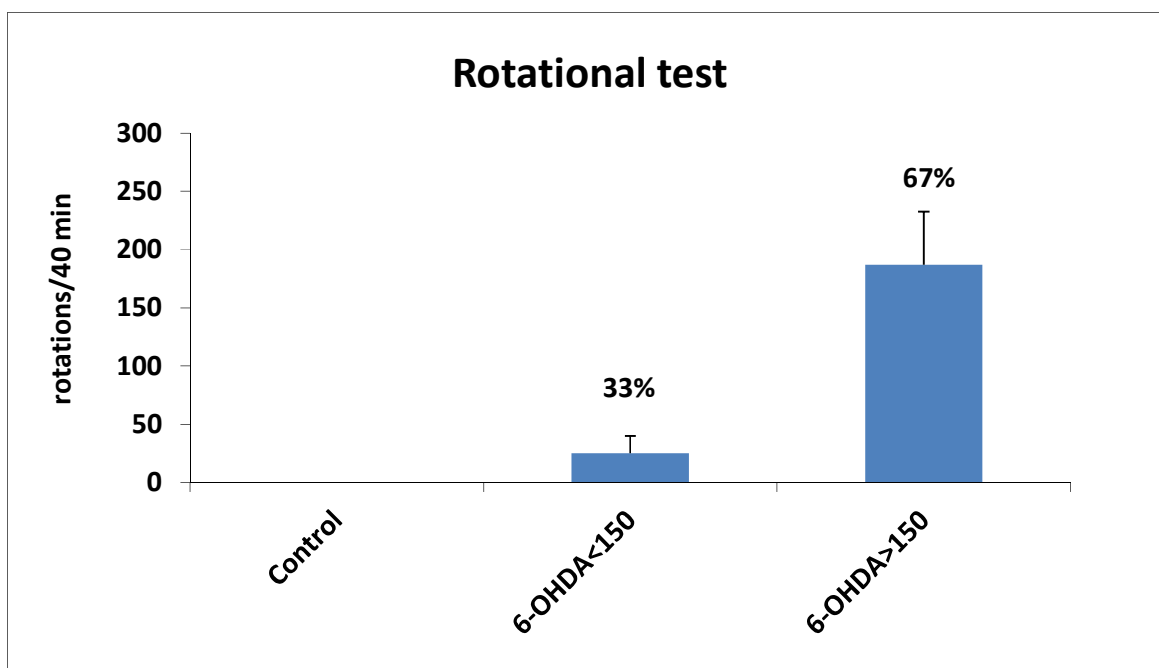


Fig.28 Behavioural test with Apomorphine.

Body weight, food consumption, fecal output, water intake and urine production of the animals belonging to the two experimental groups, hosted in metabolic cages once a week, were monitored for all the duration of the experiment (9 weeks) to assess the health condition of animals and to highlight a possible effect of the 6-OHDA lesion on these parameters.

Body growth

Body growth showed a similar pattern in 6-OHDA rats compared to control rats, although there is an initial temporary delay in the increase of weight for the lesioned rats in the period immediately after surgery. But already from the second week, despite the significant gap ($*p<0.05$) instituted, it is interesting to observe how the two growth curves are parallel (Fig. 29). For both groups, the increase in weight always remained within the standards of growth indicated by the farmer.

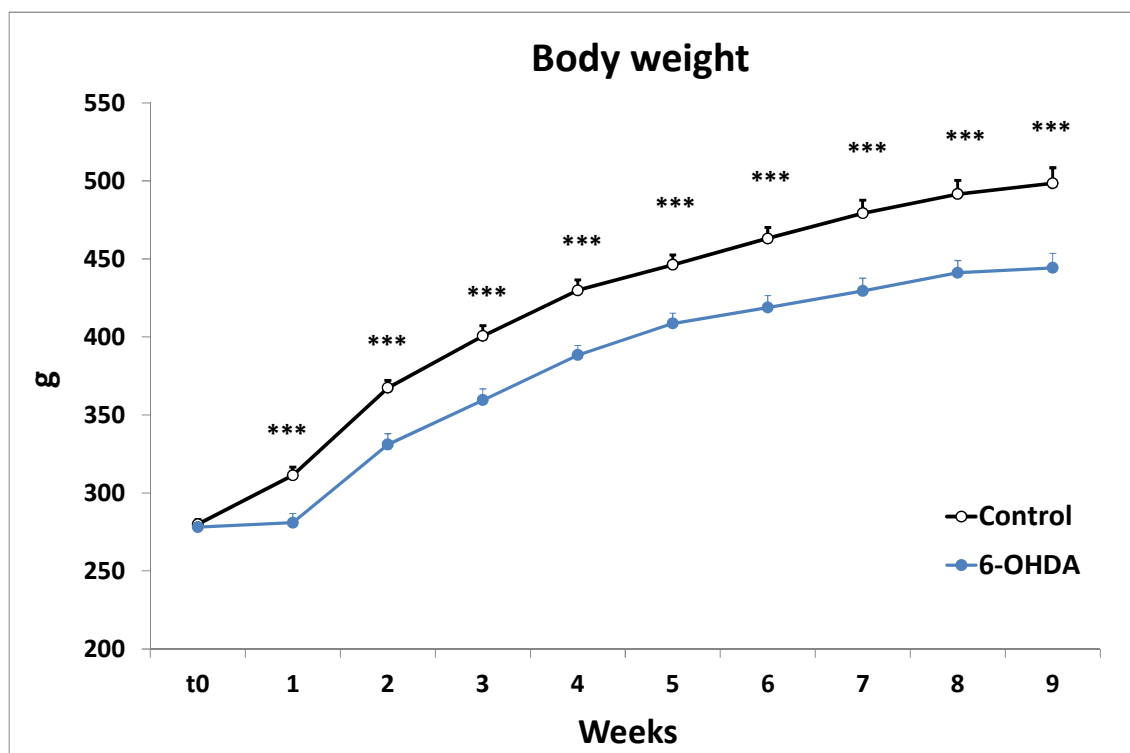


Fig. 29 Body growth of control (n=40) and 6-OHDA animals (n=44).

Food intake

The amount of food consumed in 24 hours by 6-OHDA and control rats is similar, with the exception of the first and eighth week post-lesion where the 6-OHDA rats have assumed a smaller amount of food compared to controls (* $p=0.024$ controls 27,74g vs. 6-OHDA 25g on the first week and ** $p=0.009$ controls 29,2g vs. 6-OHDA 26,46g on the eighth week). Even if not statistically significant, the 6-OHDA group starts basically to have minor food intake from the fifth week (Fig. 30).

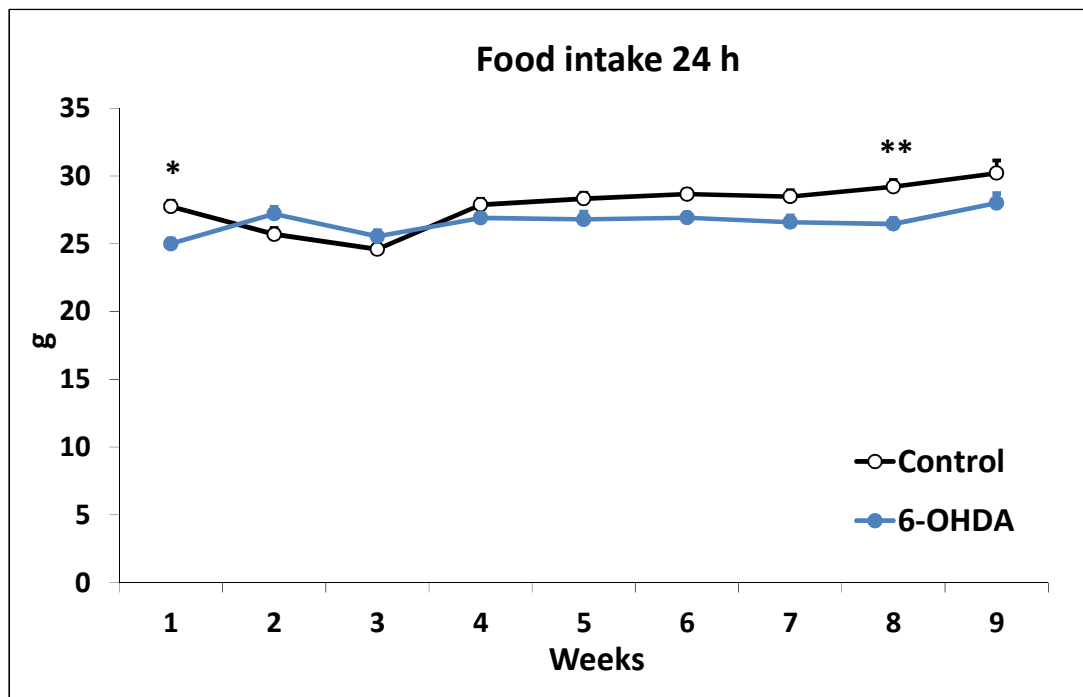


Fig. 30 Food intake in 24 h of control (n=40) and 6-OHDA animals (n=44).

Water intake and urine production

The average daily water consumption has been evaluated to highlight the presence of any alteration of the central mechanisms linked to thirst and the development of adipsia and/or dysphagia. Furthermore urine output was monitored to verify the development of urinary dysfunction, symptom often found in patients with Parkinson's disease.

As shown in Fig. 31, 6-OHDA rats drank significantly lower volumes of water compared to control animals, during all the 9 weeks of observation.

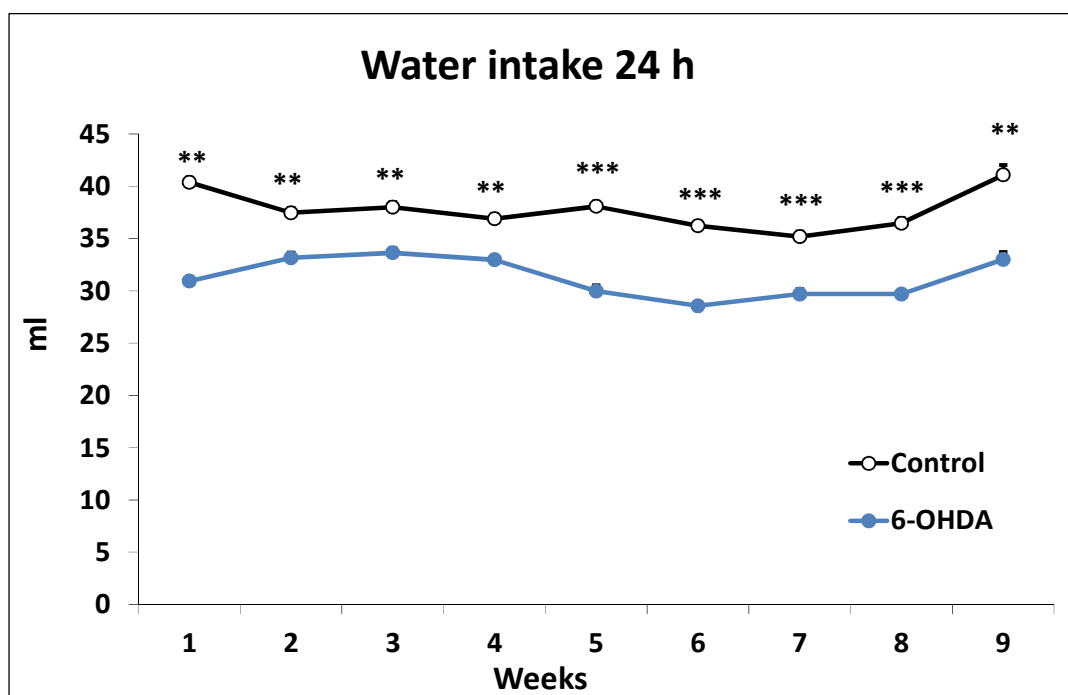


Fig. 31 Water intake in 24h of control (n=40) and 6-OHDA animals (n=44).

The average volumes of urine produced in 24 hours from 6-OHDA rats are significantly lower than those of control rats, which instead produce standard volumes. These data show an alteration of urinary behaviour that appears from the first week of observation and stays until the end (Fig.32).

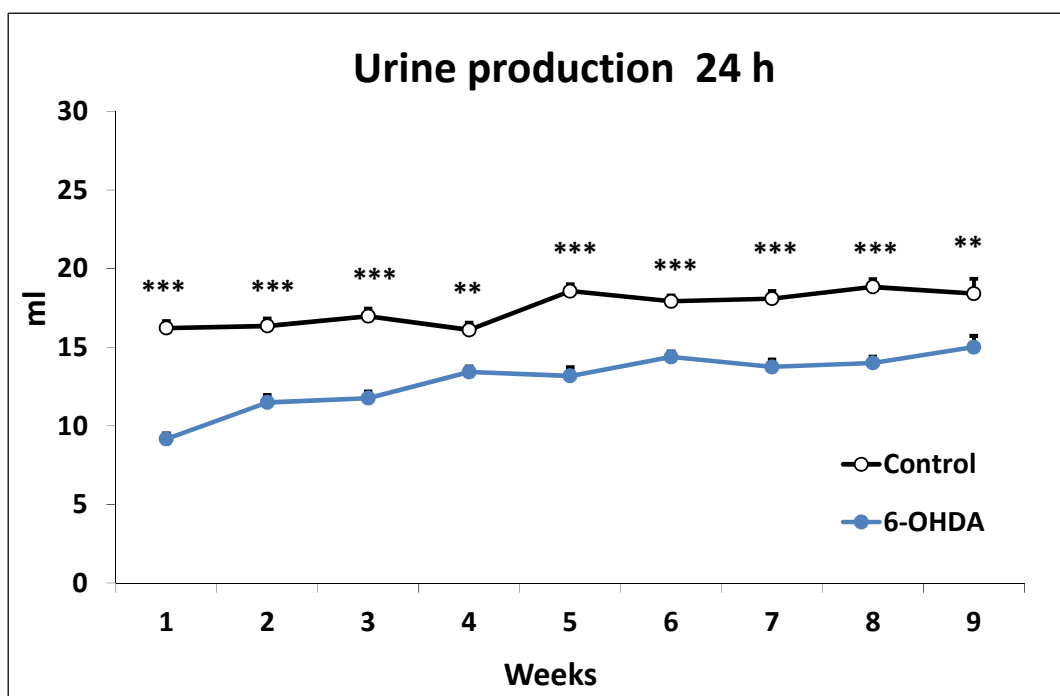


Fig. 32 Urine production in 24h of control (n=40) and 6-OHDA animals (n=44).

Fecal output and gastrointestinal transit

In order to evaluate in vivo the motor abnormalities of the gastrointestinal (GI) tract several parameters have been considered:

- Quantity of feces produced in 5 and 24 hours (fecal output), measured weekly for 8 weeks.
- Orofaecal transit, measured at the sixth and eighth week.
- Colonic motility, measured at the fourth, fifth, sixth and seventh week.
- 60 min gastrointestinal transit measured at the fourth and eighth week.
- Radiographic analysis, performed at the fourth, sixth and eighth week.

The measurement of the fecal output, carried out over 5 hours, from the third to the eighth week, allowed us to highlight a state of constipation in 6-OHDA rats. As shown in Fig. 33, the average amount of feces produced is basically decreased in 6-OHDA rats compared to control, with statistically significant values for the third, fourth and sixth weeks (* $p < 0.05$). While the amount of feces produced in 24 hours is significantly decreased only after one week from surgery (Fig. 34).

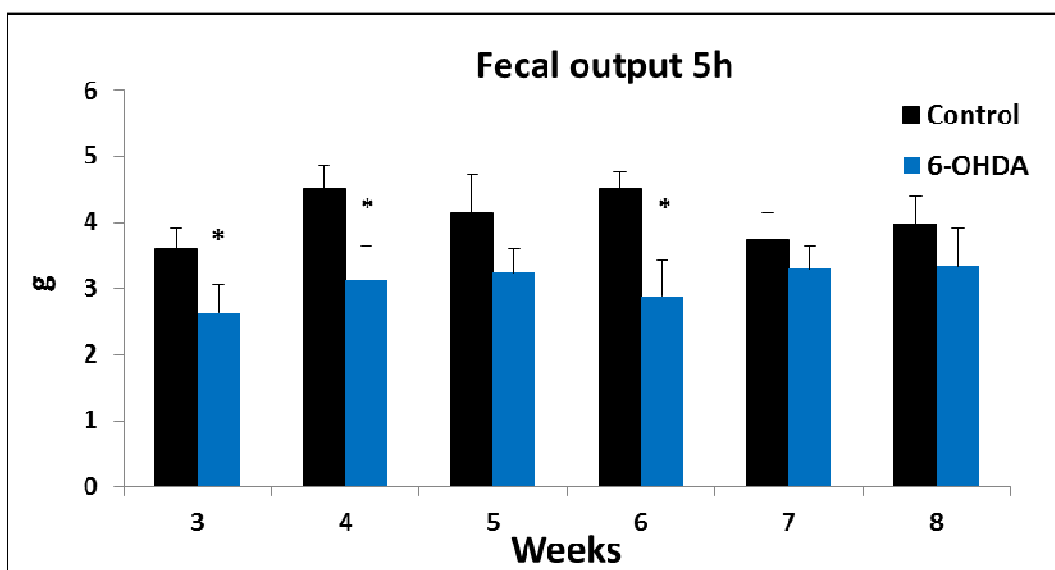


Fig. 33 Fecal output in 5h of control (n=10) and 6-OHDA (n=14) rats (t test *p<0.05)

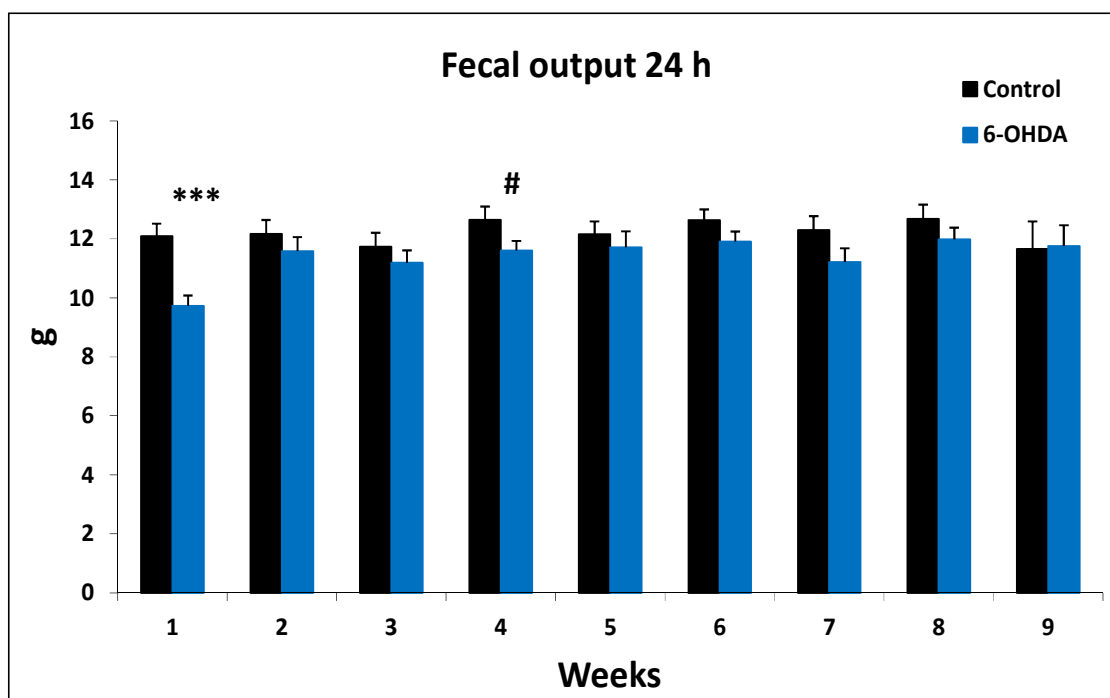


Fig. 34 Fecal output in 24h of control (n=40) and 6-OHDA rats (n=44)

(t test *p<0.05, one tailed t test #p<0.05)

Orofecal transit

The measurement of the orofecal transit, along with the fecal output and radiographic analysis (shown later), gave important information on gastrointestinal motility *in vivo*. The test performed with Evans Blue showed delayed transit in 6-OHDA than control rats. The measured values of t_2 assume significance at the sixth week for the 6-OHDA group. In fact, the average value of delay of the lesioned group compared to the control is about 52 minutes in evacuating the entire administration (t_2) (Fig.35). Repeating the test at the eighth week, the value of t_2 is still significantly higher for the 6-OHDA rats vs control ($\#p < 0.05$ Mann Whitney test), with a delay of about 35 minutes (Fig.36).

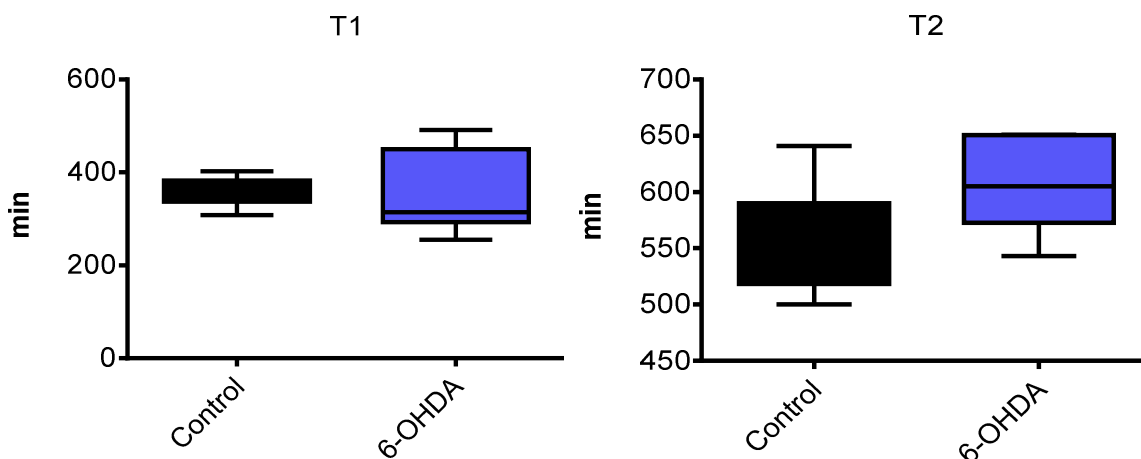


Fig. 35 Orofecal transit at the sixth week of control (n=8) and 6-OHDA rats (n=6).

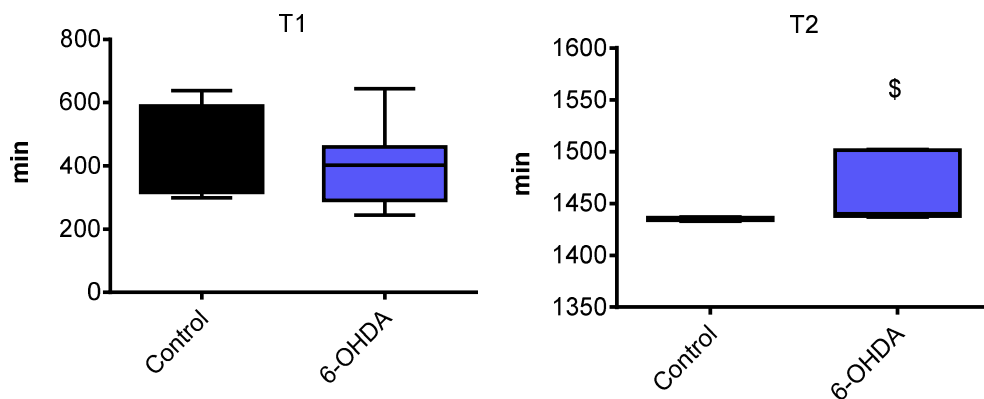


Fig. 36 Orofecal transit at the eighth week of control (n=8) and 6-OHDA rats (n=10).

\$ $p < 0.05$ Mann Whitney test

Colonic motility

The analysis of colonic motility according to the glass bead method (Raffa et al., 1987) has not shown significant differences in the transit times between the 6-OHDA group and the control. The obtained data, as shown in Fig. 37, are subject to great variability, a factor which may make the method not sufficiently sensitive to detect a colonic delay.

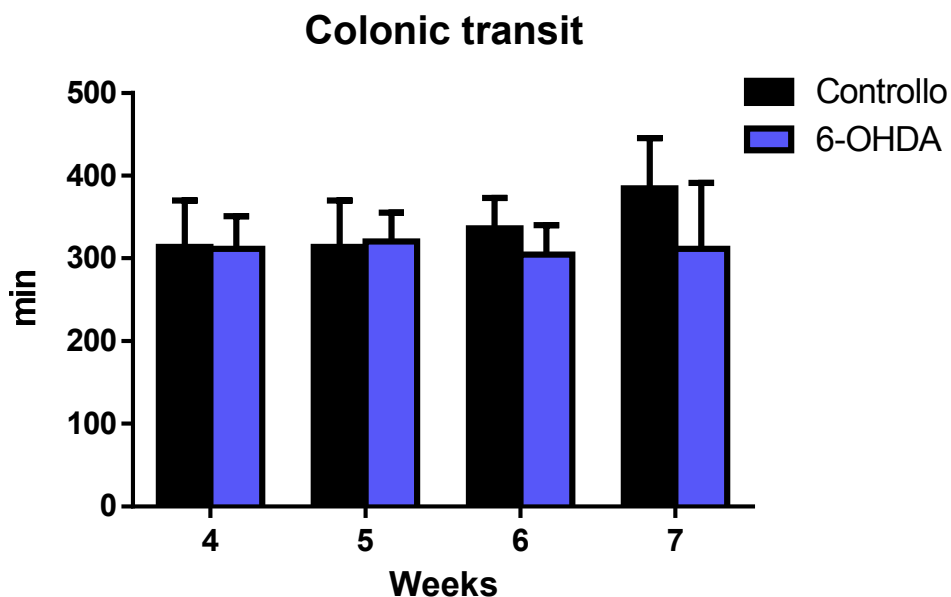


Fig.37 Bead colonic expulsion times of control (n=15) and 6-OHDA rats (n=15).

Gastrointestinal transit (De Winter method)

From the spectrophotometric analysis of samples relative to different portions of the digestive tract, it was possible to quantify the percentage of Evans Blue dye present in the individual gastrointestinal segments after 60 min from gavage and calculate the geometric centre following the method of De Winter. The geometric centre showed that the total transit in 6-OHDA rats is unchanged after 4 weeks from central lesion (Fig. 38a), but it is interestingly slowed by about 22% compared to control rats (control 7.88 ± 0.24 vs lesioned 6.12 ± 0.71) after 8 weeks (Fig. 38b).

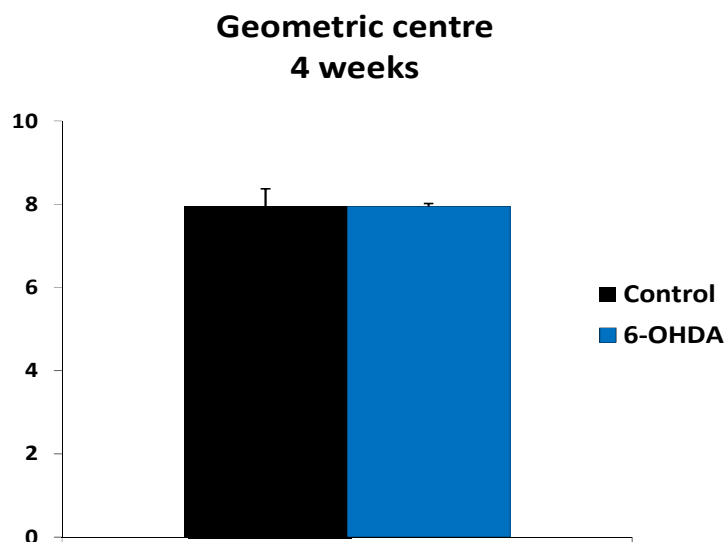


Fig. 38a Geometric centre of control (n=4) and 6-OHDA rats (n=4).

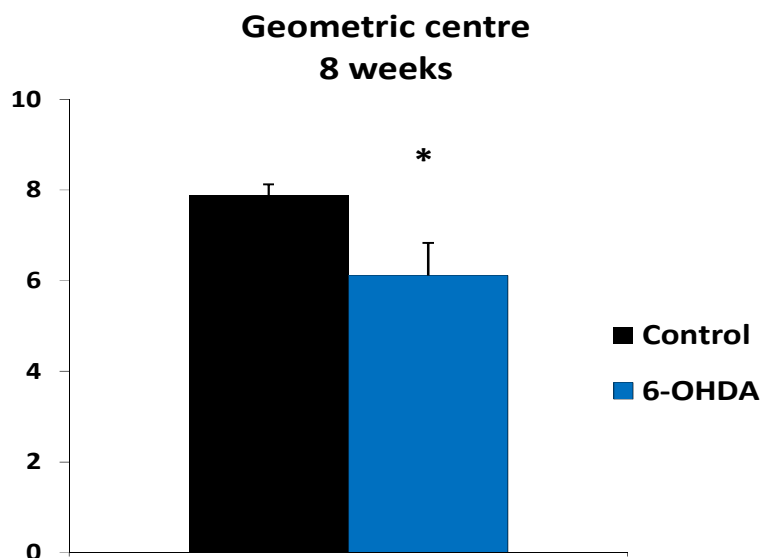


Fig. 38b Geometric centre of control (n=10) and 6-OHDA rats (n=8).

When we consider the percentages of dye present in the different analyzed segments, at 4 weeks there are no differences (Fig. 39a), but at 8 weeks we observe a delay of gastric emptying and a slower transit in ileum to distal bowel ($*p < 0.05$) in 6-OHDA rats compared to control. In fact in the stomach of the 6-OHDA animals the amount of dye (26.7%) exceeds that measured in control animals (15.5%) (Fig.39b). Furthermore, the progression of the dye in the intestine of 6-OHDA rats is slower than that in control rats. The peak of the front of progression is in the segment 8 of ileum in control rats while it stops in the segment 7 in the 6-OHDA ones. In addition there is a significantly higher percentage of dye in the segment 9 of ileum in the control ($*p < 0.05$) than in the 6-OHDA. These data

suggest the presence of a slowed motility of the stomach and intestine in 6-OHDA compared to control group, 8 weeks after central lesion.

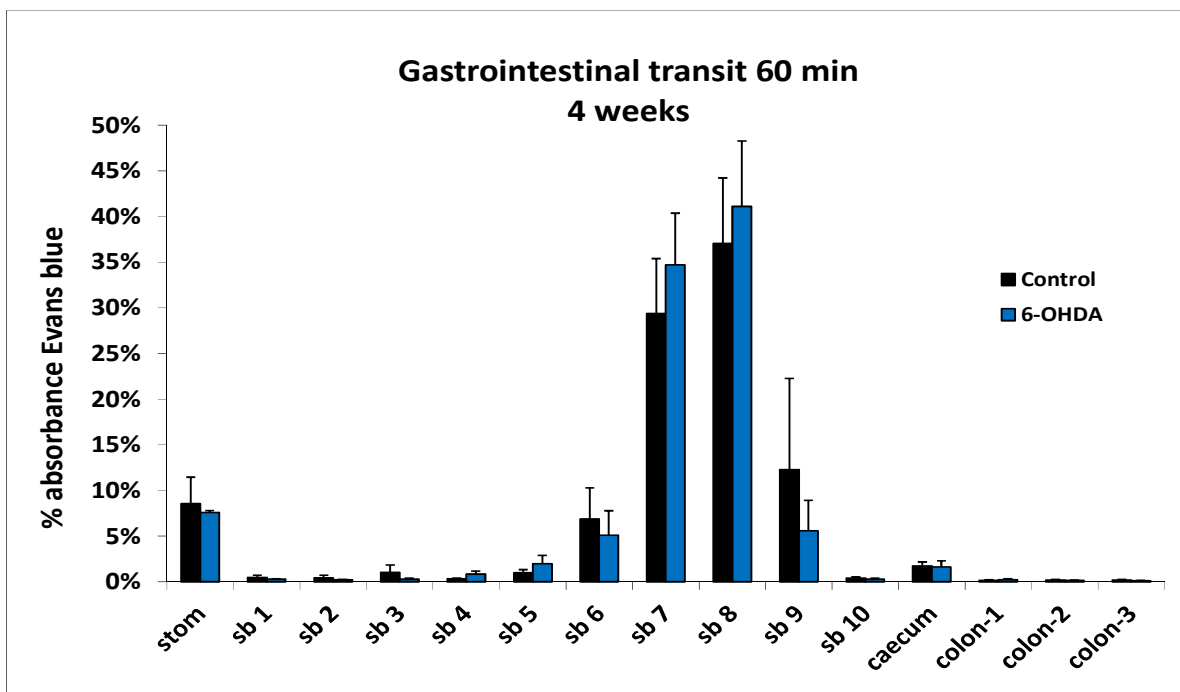


Fig. 39a Comparison between Evan Blue absorbances in the different segments of GI system in control (n=4) and 6-OHDA rats (n=4).

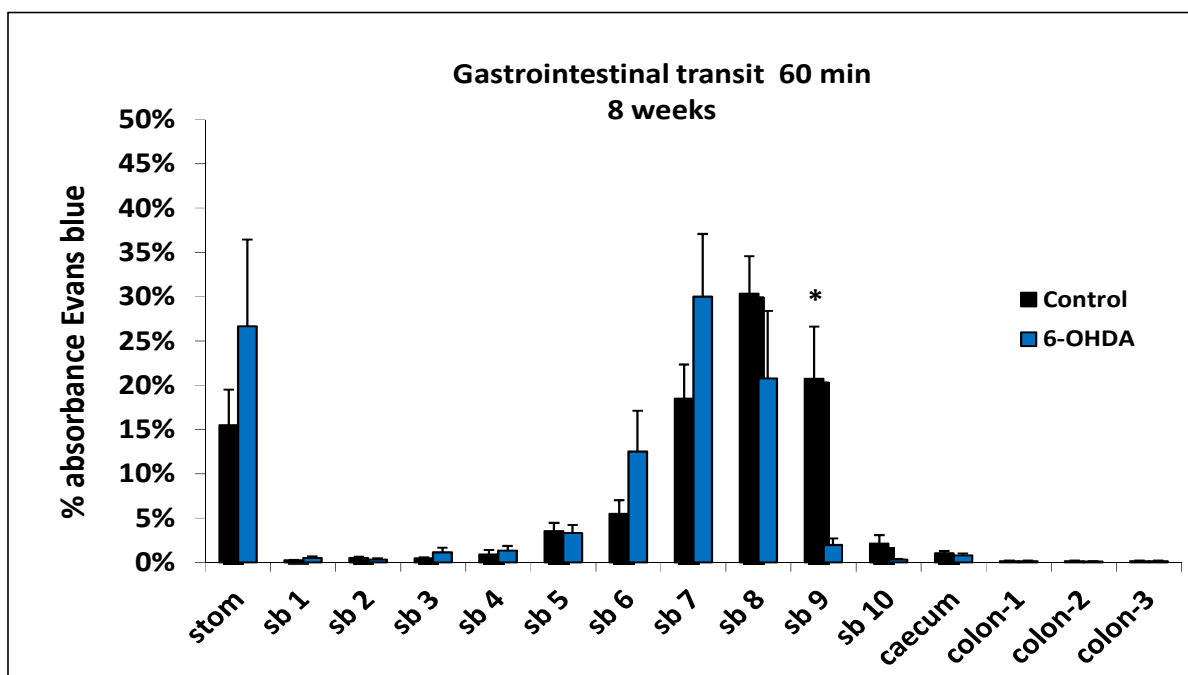


Fig. 39b Comparison between Evan Blue absorbances in the different segments of GI system in control (n=10) and 6-OHDA rats (n=8).

Radiographic analysis

To further validate and enrich the data on GI transit obtained with previous methods, it has been carried out a radiological investigation. This evaluation was repeated at the fourth, sixth and eighth week. It showed a delay in the stomach emptying phase and in the filling and emptying of the caecum and colon after eight weeks from lesion, as it can be observed in Fig. 40 and 41. Interestingly, this method has been sensitive enough to highlight also the delay in large bowel emptying, unlike the method of Raffa et al., which showed no differences in the colonic transit between 6-OHDA and control rats. From the comparative analysis of images it appears that in the caecum of 6-OHDA rats the radiopaque arrives late and persists longer than what happens in the caecum of control animals. The slow emptying of the intestine through the colonic passage, observed with this method in 6-OHDA rats, is confirmed by the extension of the t_2 period required for the complete emptying of the intestine of 6-OHDA rats compared to controls, as measured 8 weeks after surgery.

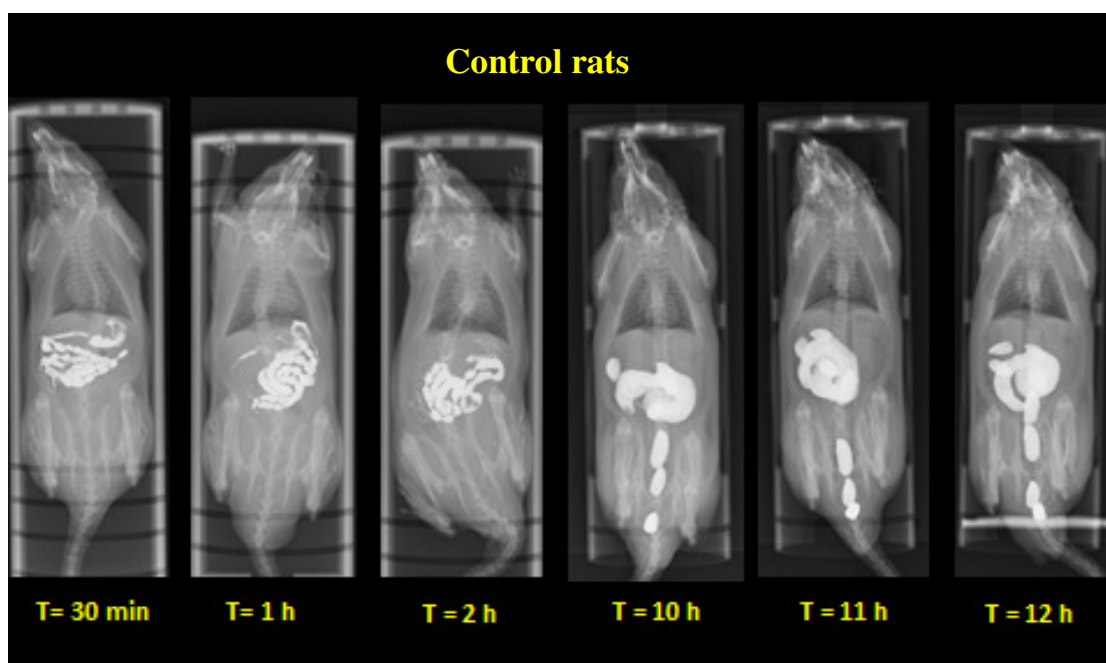


Fig. 40 Radiographic images representing the luminal passage of sulphate barium in control rats at different times, 8 weeks after surgery * (* Data published in Vegezzi G et al, 2014)

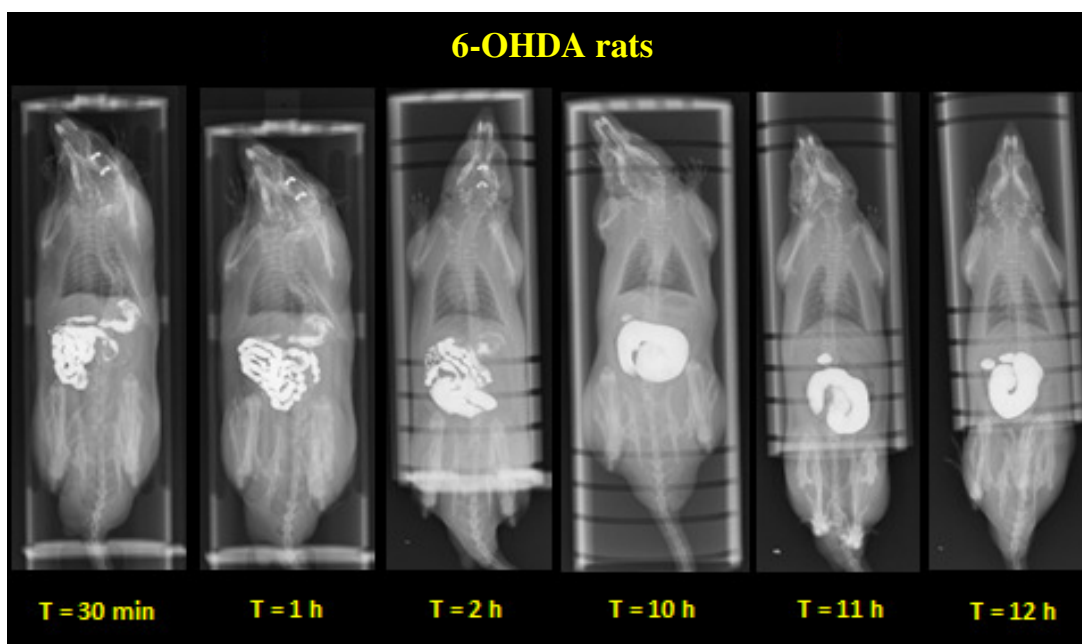


Fig. 41 Radiographic images representing the luminal passage of sulphate barium in 6-OHDA rats at different times 8 weeks after surgery *.

Following the method of Cabezos et al. 2008, the radiographic images were analyzed by blind observers, giving each portion of the GI tract a score as shown in Tables 1 and 2. To a higher score corresponds a higher content of radiopaque bolus in the various segments of the digestive tract.

Stomach	6th week					8th week				
	T=0	T=30 min	T= 1h	T= 2h	T= 10-12h	T=0	T=30 min	T= 1h	T= 2h	T= 10-12h
Control	10,9±0,3	6,3±0,3	4,0±0,4	2,0±0,3	0	10,9±0,3	6,3±0,3	4,0±0,4	2,0±0,3	0
6-OHDA	9,6±0,9	2,9±0,3	1,7±0,3	1,3±0,2	0	11,3±0,3	7,3±0,2	5,4±0,5	3,2±1,4	0
6-OHDA+L-DOPA/ Benserazide	10,1±0,5	3,7±1,9	2,5±1,7	0,9±0,1	0	10,4±0	5,2±1,0	3,8±0,5	1,6±0,5	0

Small intestine	6th week					8th week				
	T=0	T=30 min	T= 1h	T= 2h	T= 10-12h	T=0	T=30 min	T= 1h	T= 2h	T= 10-12h
Control	5,9±0,3	9,1±0,2	9,8±0,2	9,7±0,2	0	5,9±0,3	9,1±0,2	9,8±0,2	9,7±0,2	0
6-OHDA	8,0±0,4	10,6±0,3	10,4±0,7	9,9±0,1	0	4,3±1,6	9,6±0,4	10,1±0,2	4,4±2,2	0
6-OHDA+L-DOPA/ Benserazide	6,6±0,8	9,2±0,7	10,1±0,4	7,5±1,2	0	5±0,4	10,3±0,9	10,5±0,3	9±0,7	0

Table 1. Score values of stomach and small intestine in control (n=20), 6-OHDA (n=20) and 6-OHDA/treated rats (n=6)*.

From the values shown in Table 1 it is possible to observe, at 6 weeks from brain damage, an accelerated gastric emptying and ready filling of the small intestine within 2 hours from radiopaque gavage in 6-OHDA rats compared to controls. Similar behaviour is shown by 6-OHDA animals

treated for 2 weeks with L-DOPA/Benserazide. The picture changes when the radiological analysis was repeated at 8 weeks after the central lesion. As already anticipated, 6-OHDA animals present a delayed gastric emptying, which is no more present in 6-OHDA animals treated with L-DOPA/Benserazide.

Caecum						8th week				
6th week						8th week				
	T=0-1h	T=30 min	T= 1h	T= 2h	T= 10-12h	T=0	T=30 min	T= 1h	T= 2h	T= 10-12h
Control	0	0,4±0,4	10,5±0,4	9,7±0,5	9,3±0,5	0	0,4±0,4	10,5±0,4	9,7±0,5	9,3±0,5
6-OHDA	0	0	9,7±0,6	9,1±1,0	8,7±1,0	0	0	6,7±1,3	7,2±1,4	7,5±0,8
6-OHDA+L-DOPA/ Benserazide	0	0	7,2±6,8	7,6±6,3	6,5±7,0	0	0	11,8±0,1	10,9±1,5	11±0,7

Colon						8th week				
6th week						8th week				
	T=0-1h	T=30 min	T= 1h	T= 2h	T= 10-12h	T=0	T=30 min	T= 1h	T= 2h	T= 10-12h
Control	0	0	7,9±0,6	8,8±0,7	8,1±1,1	0	0	7,9±0,6	8,8±0,7	8,1±1,1
6-OHDA	0	0	2,5±1,9	9,3±1,1	8,6±1,4	0	0	2,4±0,9	2,6±1,0	5,1±1,0
6-OHDA+L-DOPA/ Benserazide	0	0	6,6±2,9	8,1±1,8	7±2,1	0	0	2,5±2,5	5,6±1,5	6,5±2,2

Table 2. Score values of caecum and colon in control (n=20), 6-OHDA (n=20) and 6-OHDA/ treated rats (n=6)*.

The values shown in Table 2, at six weeks, show no significant differences in emptying time of caecum among 6-OHDA, control and treated with L-Dopa/Benserazide groups while emerges a late filling of the colon in the group of 6-OHDA animals. At the 8th week, the delay in the filling of the caecum and colorectum displayed by 6-OHDA group compared to the control appears partially prevented by the four weeks pharmacological treatment.

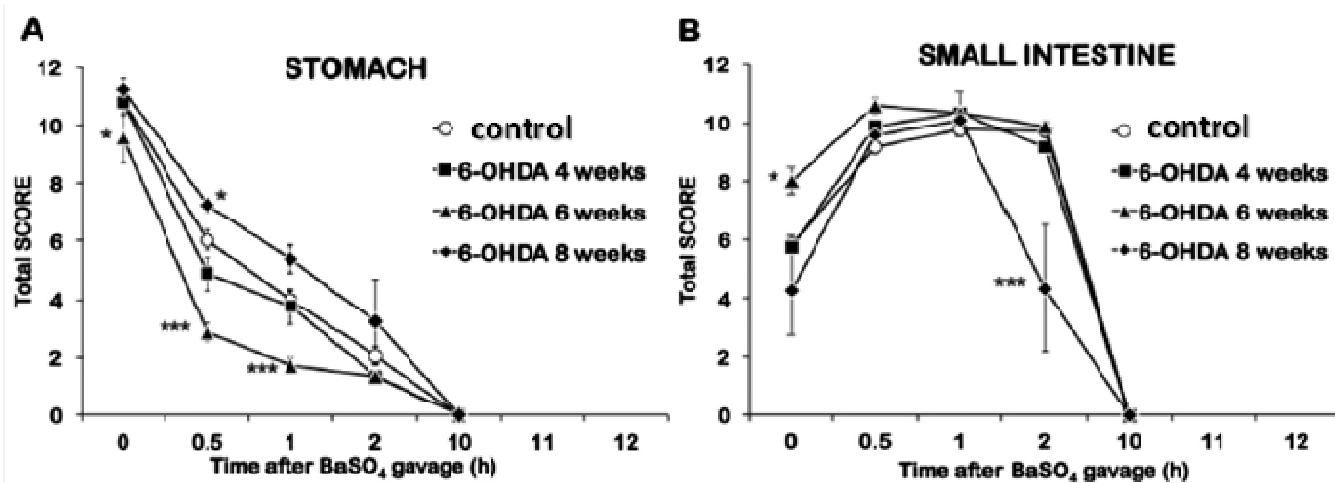


Fig. 42a Graphic representations of the radiologic analysis 4, 6 and 8 weeks after 6-OHDA injection. The total score was assessed at 0, 0.5, 1, 2, 10, 11 and 12 h after barium sulphate gavage, in the stomach (A) and small intestine (B). * $p < 0.05$, *** $p < 0.001$ vs control*.

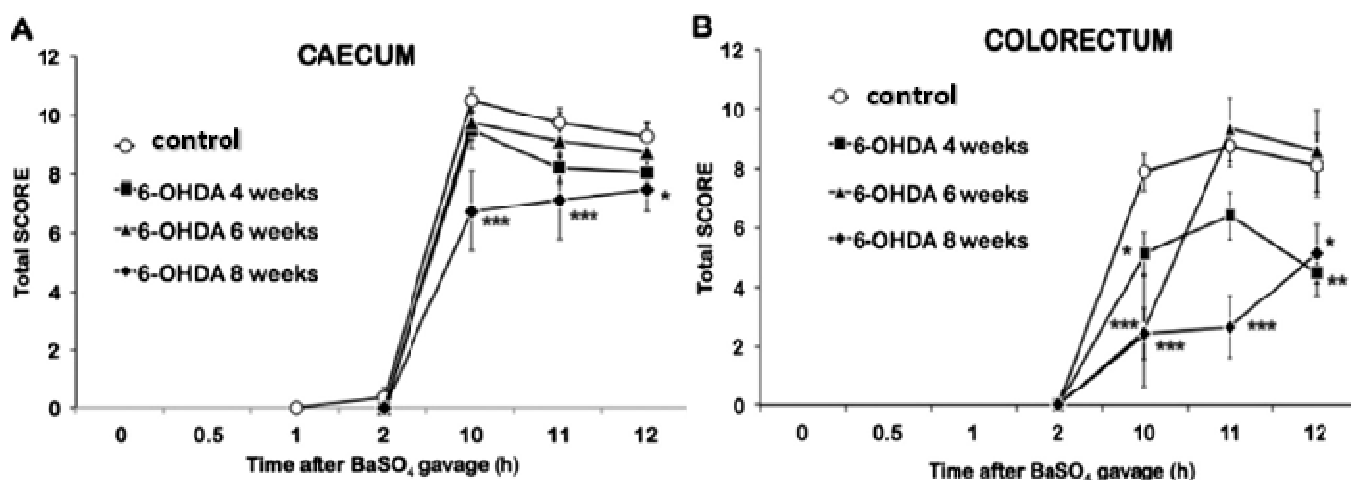


Fig. 42b Graphic representations of the radiologic analysis 4, 6 and 8 weeks after 6-OHDA injection. The total score was assessed at 0, 0.5, 1, 2, 10, 11 and 12 h after barium sulphate gavage, in the caecum (A) and colorectum (B). * $p < 0.05$, *** $p < 0.001$ vs control*.

Part 2: Effects of oral subchronic treatment with L-DOPA / Benserazide

The oral subchronic treatment with L-DOPA/Benserazide (6mg/kg, 15mg/kg) reduces the difference in body weight instituted between the 6-OHDA and control rats, but just in small extent since the difference remains anyways statistically significant ($*p < 0.05$ Control vs 6-OHDA/ treated) (Fig.43).

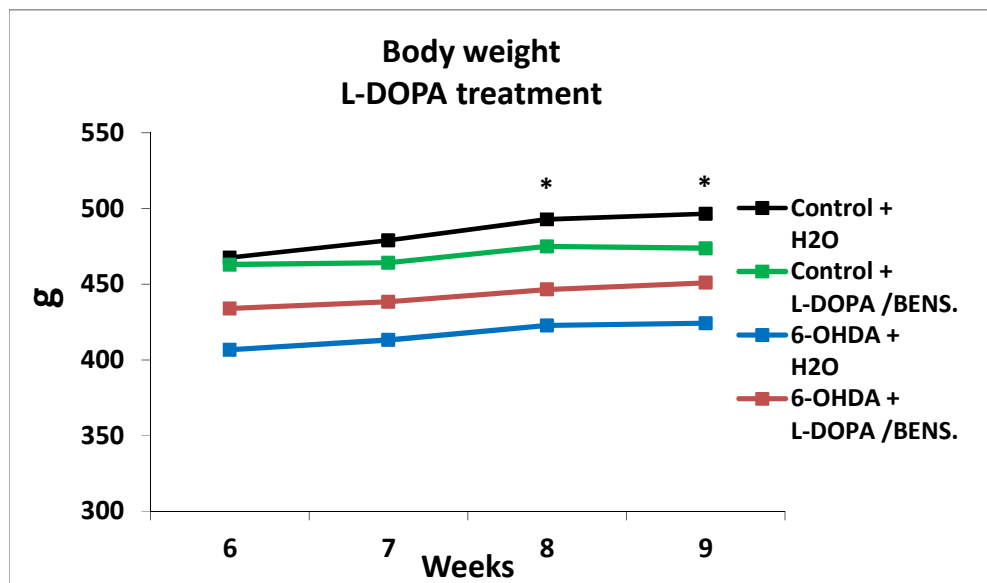


Fig. 43 Body weight of control (n=6), control/treated (n=6), 6-OHDA (n=10) and 6-OHDA/ treated rats (n=10).

The treatment with L-DOPA/Benserazide does not seem to affect the daily intake of food in the different groups (Fig. 44).

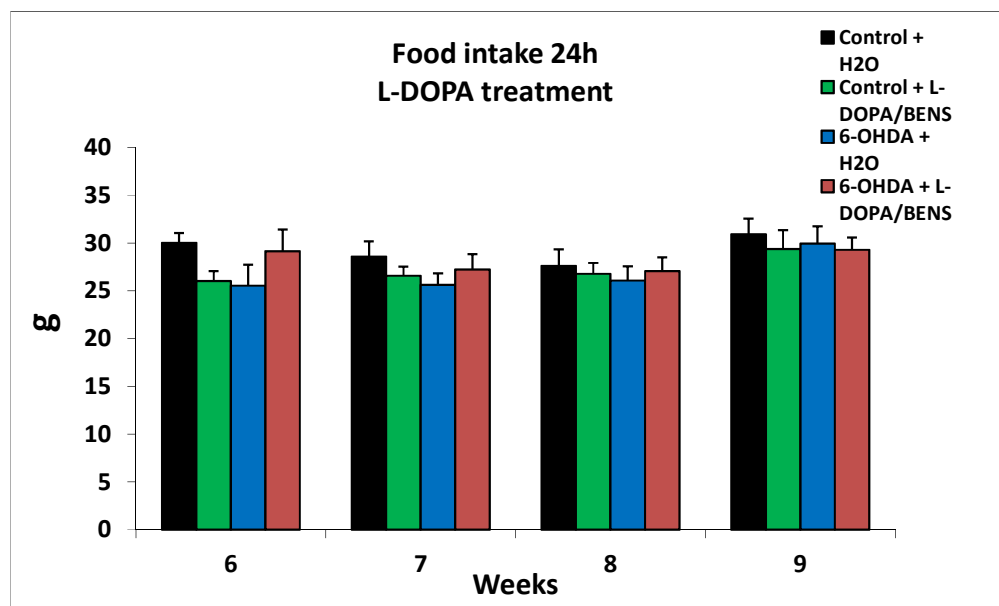


Fig. 44 Food intake of control (n=6), control/treated (n=6), 6-OHDA (n=10) and 6-OHDA/ treated rats (n=10).

As can be observed from the graph in Fig. 45 concerning a subset of animals, besides the fact that 6-OHDA rats tend to produce a daily quantity of feces lower than the control in the fifth, sixth and eighth week, the treatment with L-DOPA/Benserazide seems to provide an improvement of this condition although with discontinuity (Fig.45).

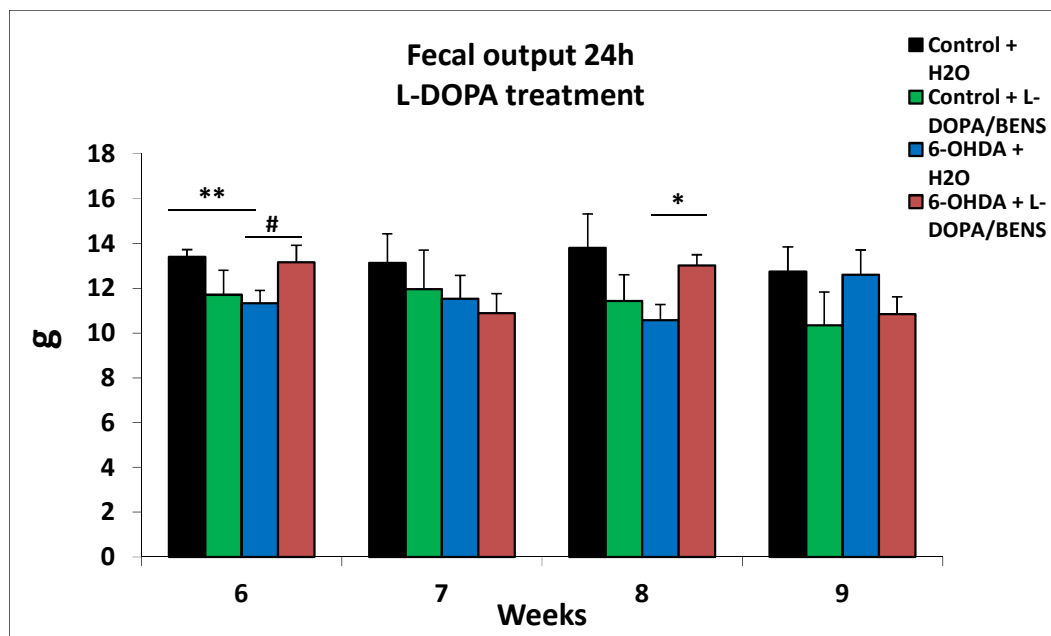


Fig. 45 Fecal output of control (n=6), control/treated (n=6), 6-OHDA (n=10) and 6-OHDA/ treated rats (n=10).

It seems that after one, two and four weeks of daily oral subchronic treatment with L-DOPA/ Benserazide, the volume of water consumed in 24 hours from the 6-OHDA/treated rats is maintained significantly lower (* p < 0.05; ** p < 0.01) compared to control (Fig. 46).

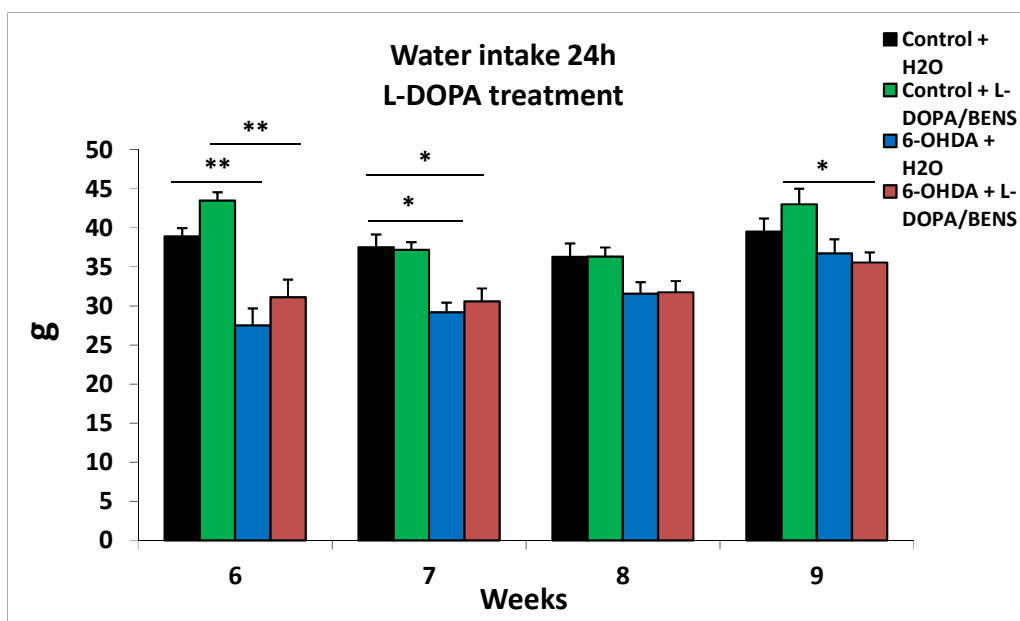


Fig. 46 Water intake of control (n=6), control/treated (n=6), 6-OHDA (n=10) and 6-OHDA/ treated rats (n=10).

For the whole period of observation the 6-OHDA/treated group has produced a daily volume of urine significantly lower (** p < 0.01; *** p < 0.001) compared to control group (Fig. 47).

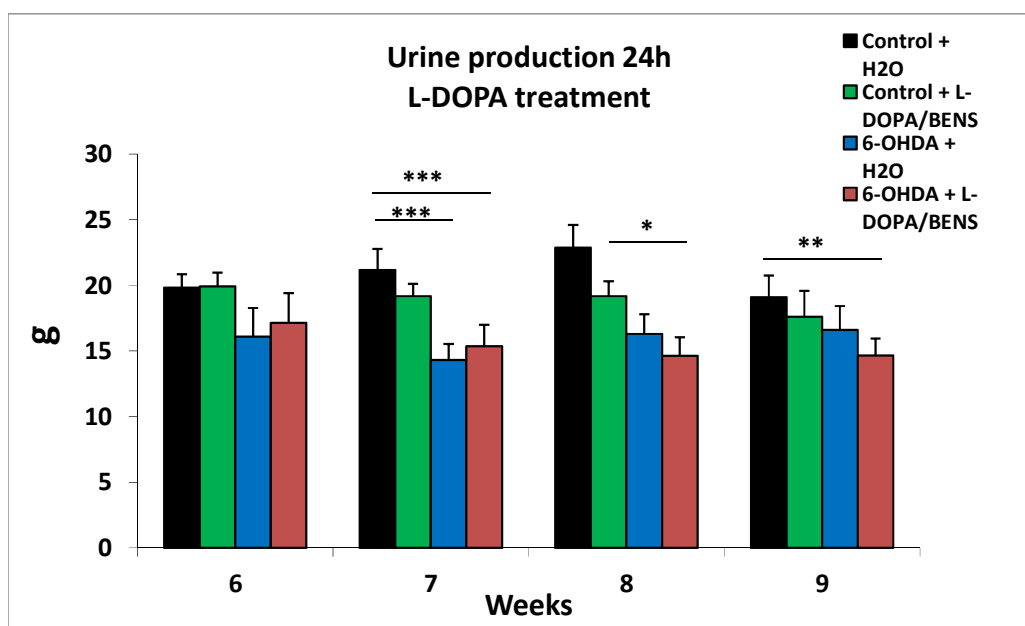


Fig. 47 Urine production of control (n=6), control/treated (n=6), 6-OHDA (n=10) and 6-OHDA/ treated rats (n=10).

In order to put in evidence the effects of treatment with L-DOPA/Benserazide on gastrointestinal transit speed, the progression profiles of the dye have been compared in the various segments of the GI for each experimental group. In the portion 8 and 9 of ileum there is a significant ($*p<0.05$, $**p<0.01$) higher amount of dye in the control group compared to the 6-OHDA/treated (Fig. 48). The analysis of the geometric centre, despite the presence of basically lower values for 6-OHDA/treated group, did not show a significant differences compared to control or 6-OHDA rats (Fig. 49).

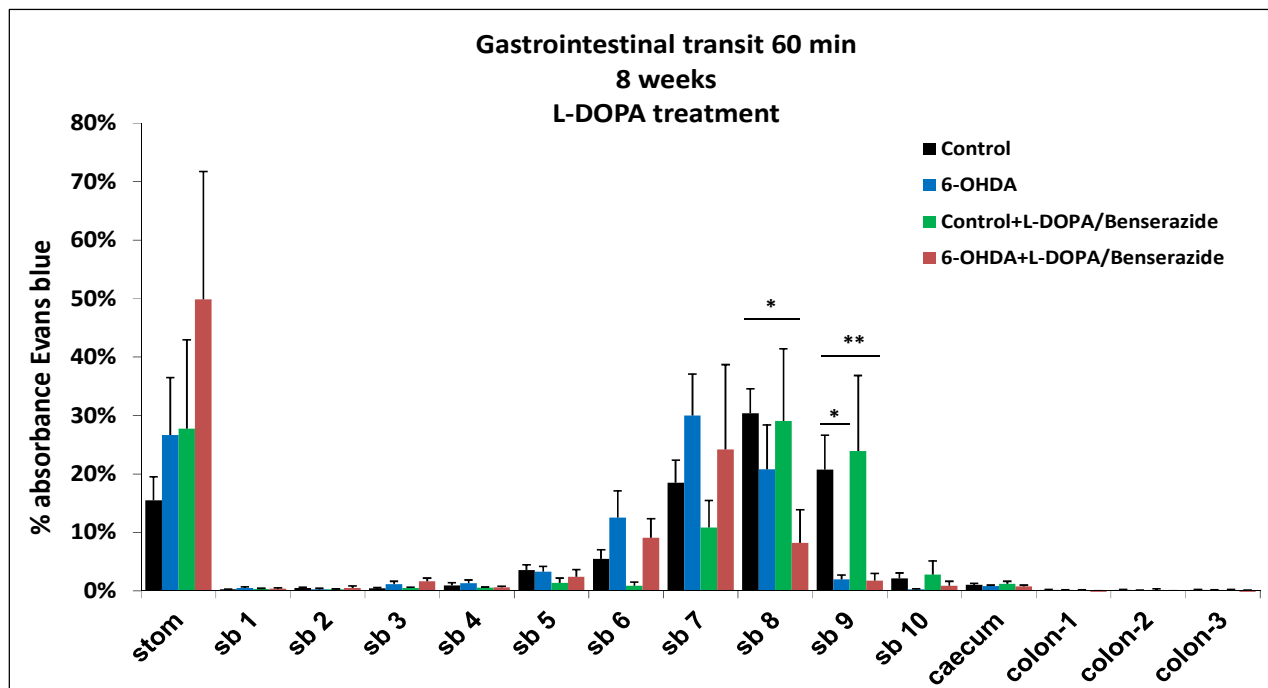


Fig. 48 Comparison between Evan Blue absorbances in the different segments of GI system in control (n=10), control/treated (n=4), 6-OHDA (n=8) and 6-OHDA/treated (n=4).

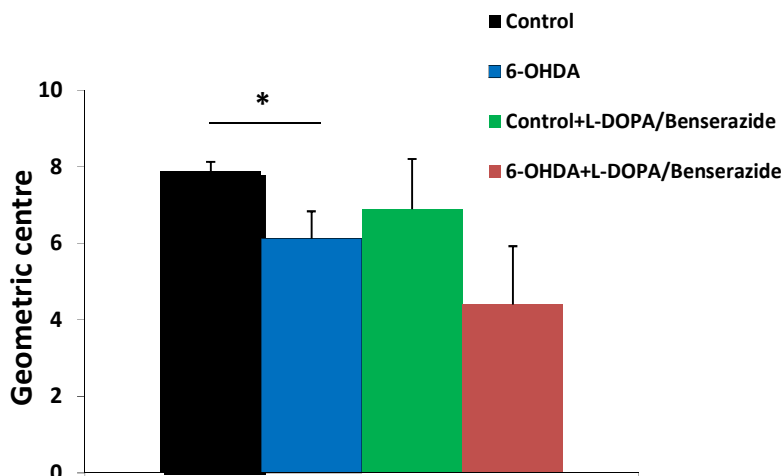


Fig. 49 Geometric centre of control (n=10), control/treated (n=4), 6-OHDA (n=8) and 6-OHDA/treated (n=4).

Comparing 6-OHDA group with 6-OHDA/treated group, in the stomach of the second group there is a greater colorant quantity than that measured in untreated animals (49.86% treated vs 26.67% untreated) and only in the portion 8 of the ileum the progression of the dye in treated rats is more clearly less than that in the untreated rats (8.18 % treated vs. 20.78 % untreated) (Fig. 48).

The geometric centre shows a general slowing of total transit in 6-OHDA/treated rats compared to only 6-OHDA (6-OHDA/L-DOPA 4.40 ± 1.53 vs 6-OHDA 6.12 ± 0.71) even if does not reach significance levels (Fig. 49).

From a comparison of radiographic images related to the 6-OHDA rats and those receiving the subchronic treatment with L-DOPA/benserazide is possible to observe how such treatment prevented the delayed gastric emptying and partially reversed the delayed progression of the luminal contents in the caecum and colon of 6-OHDA animals.

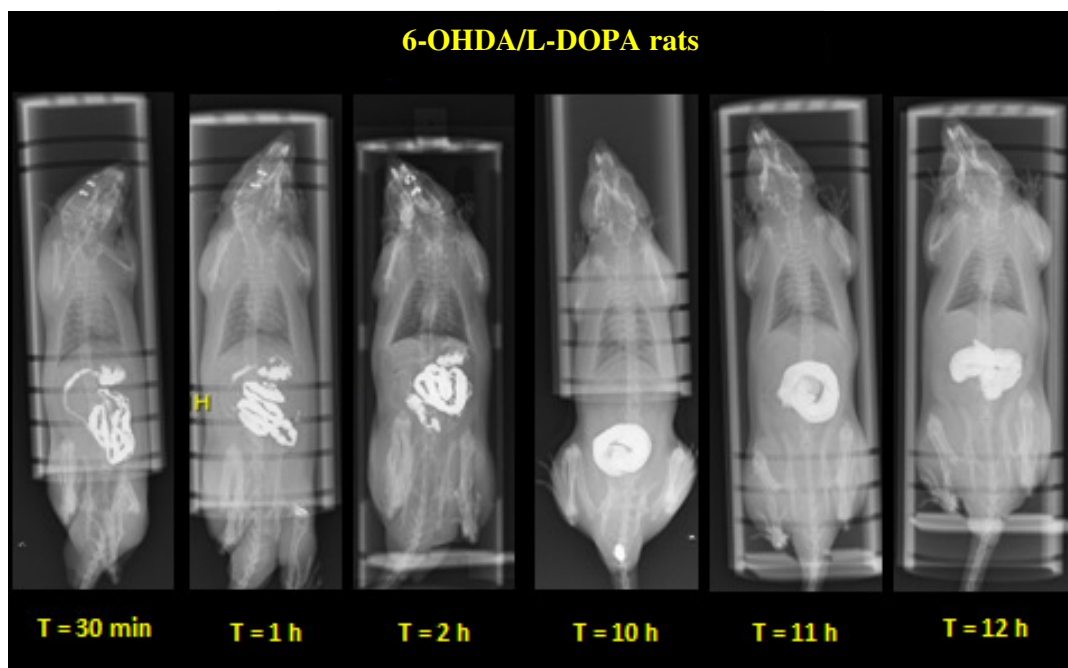


Fig. 50 Radiographic images representing the luminal passage of sulphate barium in 6-OHDA rats treated for 4 weeks with L-DOPA/Benserazide at different times.

In vitro organ bath functional assays (8 weeks)

Circular strip of gastric fundus

Pharmacological stimulation

• Dose-response curve to bethanechol

In the circular muscle strip of gastric fundus isolated from 6-OHDA animals, the pharmacological stimulation by bethanechol generated a significantly lower response compared to the corresponding control tissue. The muscarinic agonist has indeed produced a maximum effect approximately halved, compared to that observed for control rats (Emax Control 51.9 g/g tissue - Emax 6-OHDA 24.2 g/g tissue [t-test = 0.031 - * p < 0.05]) (Fig. 51).

Agonist potency remained almost unchanged in both conditions, as evidenced by the similar values of the respective EC₅₀ (pD₂ Control 5.44 vs pD₂ 6-OHDA 5.31). These data suggest that, at this level, the sensitivity of 6-OHDA rats to cholinergic stimulation is compromised. This feature is originally reported in this work. Indeed in this model other researchers described no variation in gastric acetylcholine content or ChAT expression (Colucci, 2012; Zhu 2012) 4 weeks after 6-OHDA microinjection while Zheng et al. (2011) reported a reduced content of acetylcholine in the muscularis externa of the stomach of rats bilaterally lesioned 6 weeks before.

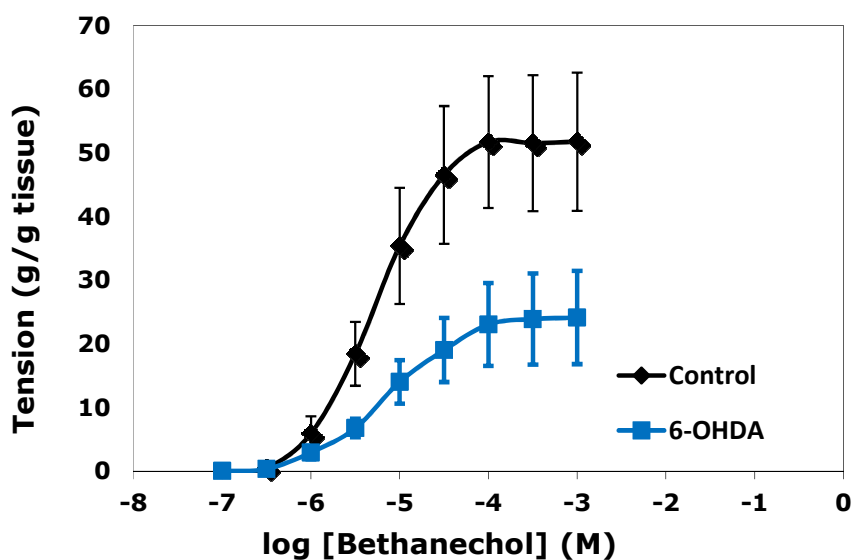


Fig. 51 Dose-response curve to bethanechol in control (n=7) and 6-OHDA rats (n=8) (circular strip of gastric fundus).

• *Curve concentrazione-risposta da PGF2 α*

Even the response of 6-OHDA gastric fundus to the application of increasing concentrations of PGF2 α has proved to be lower than that of the control tissue, recording a halving of the maximum effect (Emax Control 20.85 g/gtissue - Emax 6-OHDA 10.16 g/gtissue [t-test = 0153]) (Fig. 52), although not significant in statistical terms, probably due to a non-negligible variability.

Also in this case, the EC50 are very similar to each other (pD2 Control 6.13 - pD2 6-OHDA 6.17). The Prostaglandin F2alpha induces contraction of gastrointestinal smooth muscle: along with serotonin, it results in a concentration-dependent contractions of fundus gastric strips of rats with an intracellular mechanism distinct from that due to the muscarinic stimulation, sensitive to calcium blockers and independent of phosphoinositide hydrolysis (Secrest et al., 1989).

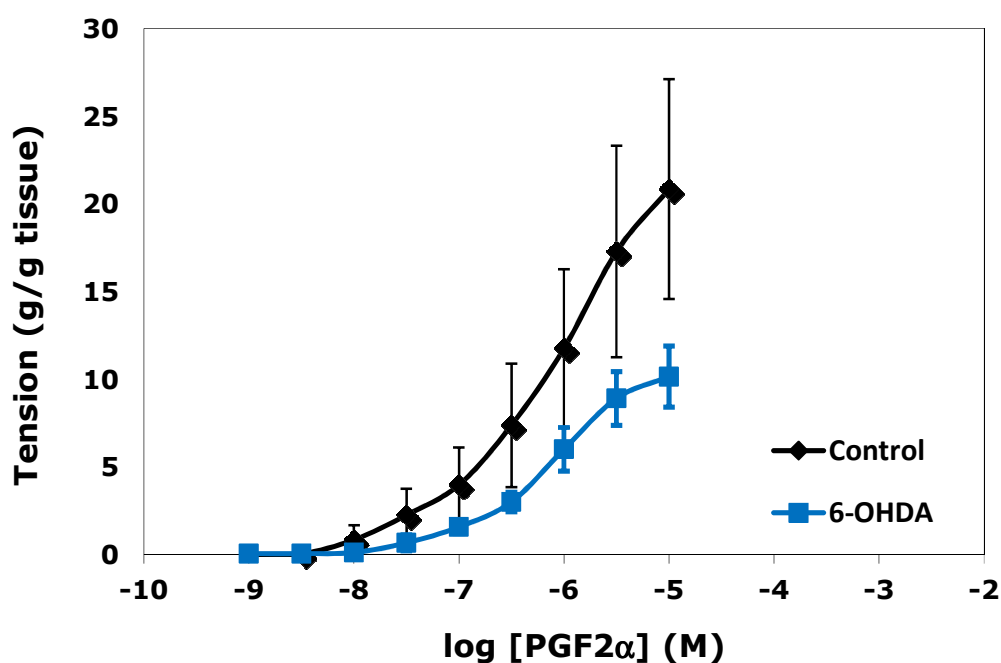


Fig. 52 Dose-response curve to PGF-2 α in control (n=7) and 6-OHDA rats (n=8) (circular strip of gastric fundus).

• *Relaxation curves by Dopamine*

On a hypertone produced by administration of an appropriate concentration of PGF2 α (corresponding to the EC50 obtained from the previous dose-response curve) cumulative increasing concentrations of dopamine were applied. Dopamine caused in the 6-OHDA gastric fundus strip a relaxation seemingly minor compared to control tissue, although the difference in term of potency was non-statistically significant (t-test [pD2 Control vs. 6-OHDA] p= 0.815) (Fig. 53).

This data would indicate the presence of an alteration in 6-OHDA animals of the response mediated by dopamine receptors at the level of gastric tissue. Alterations in the function of the dopaminergic system have emerged also in the immunohistochemical studies reported by Tian (2008), which described an increase in the expression of the neuronal dopamine transporter DAT and of the enzyme involved in its synthesis (tyrosine hydroxylase, TH) in epithelial cells and neurons of the stomach, duodenum and colon of rats lesioned with 6-OHDA by four weeks.

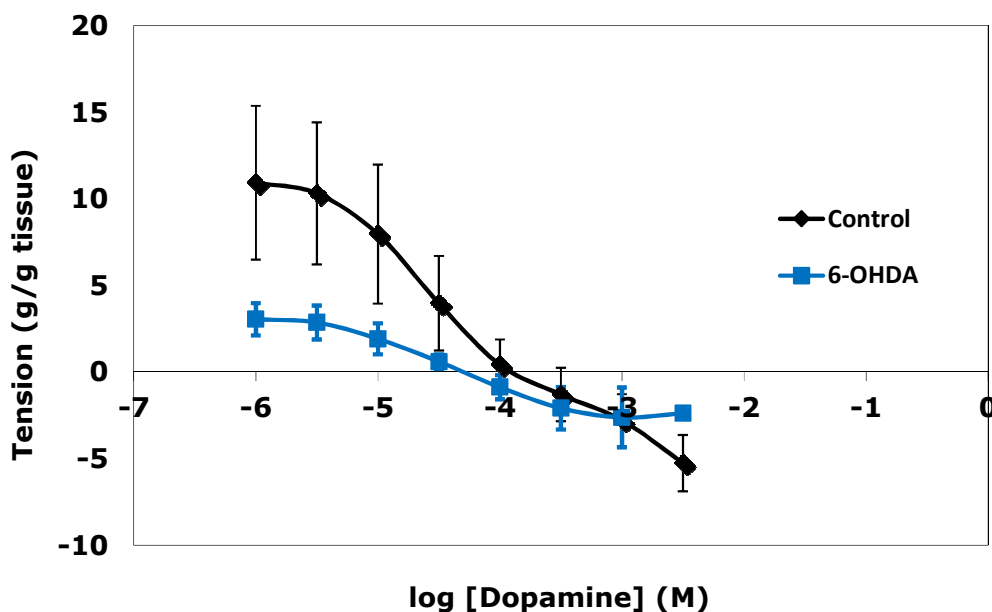


Fig. 53 Dose-response curve to Dopamine applied on PGF2a-induced hypertone in control (n=7) and 6-OHDA rats (n=8) (circular strip of gastric fundus).

It is also important to note that the dopamine-relaxing activity was inhibited by the presence of the D2/D3 receptor antagonist Sulpiride, both in 6-OHDA and control animals.

In Figure 54 it is evident how the antagonist at the concentration of 10^{-6} M in both cases has lowered the maximum response, without producing any appreciable rightward shift of the dose-response curve (E_{max} control and 6-OHDA in the absence of antagonist=100.0% - E_{max} Control in the presence of antagonist=60.6%/ E_{max} 6-OHDA in the presence of antagonist=65.2%). This may be indicative of the involvement of D2 receptors, which represent the most expressed subtype in the gastric mucosa of rats (Wang et al., 2011), in the inhibitory response by dopamine on the muscles of the gastric fundus.

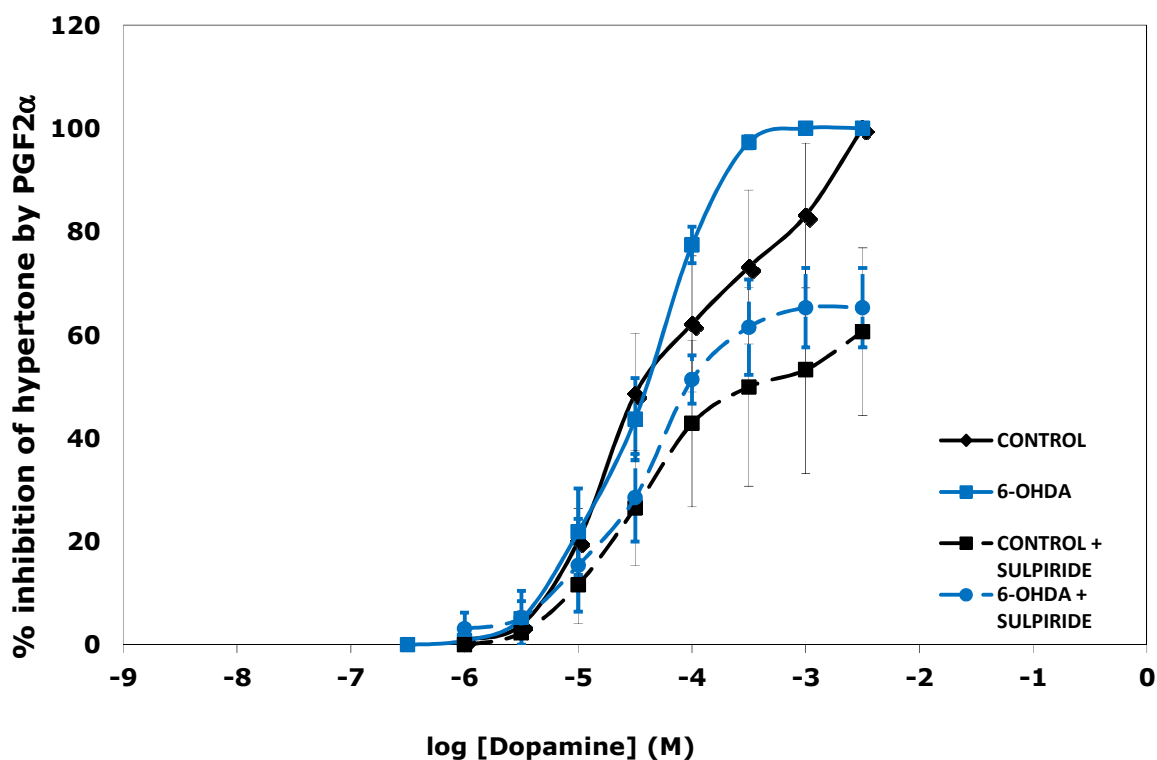


Fig. 54 Percentage of inhibition of the PGF2 α -induced hypertone by Dopamine in the presence of Sulpiride in control (n=7) and 6-OHDA rats (n=8) (circular strip of gastric fundus).

• *Relaxation curves by Dopamine in absence of nitreergic contribution*

The relaxant responses to dopamine have been determined also after the addition of NO synthase inhibitor (L-NAME, 3×10^{-4} M) in order to eliminate the possible relaxant effect resulting from the endogenous NO. As it visible from Figure 55, the data suggest that even in this case control tissues tend to relax more than 6-OHDA tissues: within the range of concentrations of dopamine used there are no significant changes compared to the previous condition (in the absence of L-NAME) for both control and 6-OHDA tissues.

Relaxation of control tissue: from 10.87 to -3.15 g/g tissue; Relaxation of 6-OHDA tissue: from 3.49 to 1.29 g/g tissue; t-test [pD2 control vs pD2 6-OHDA] $p = 0.667$.

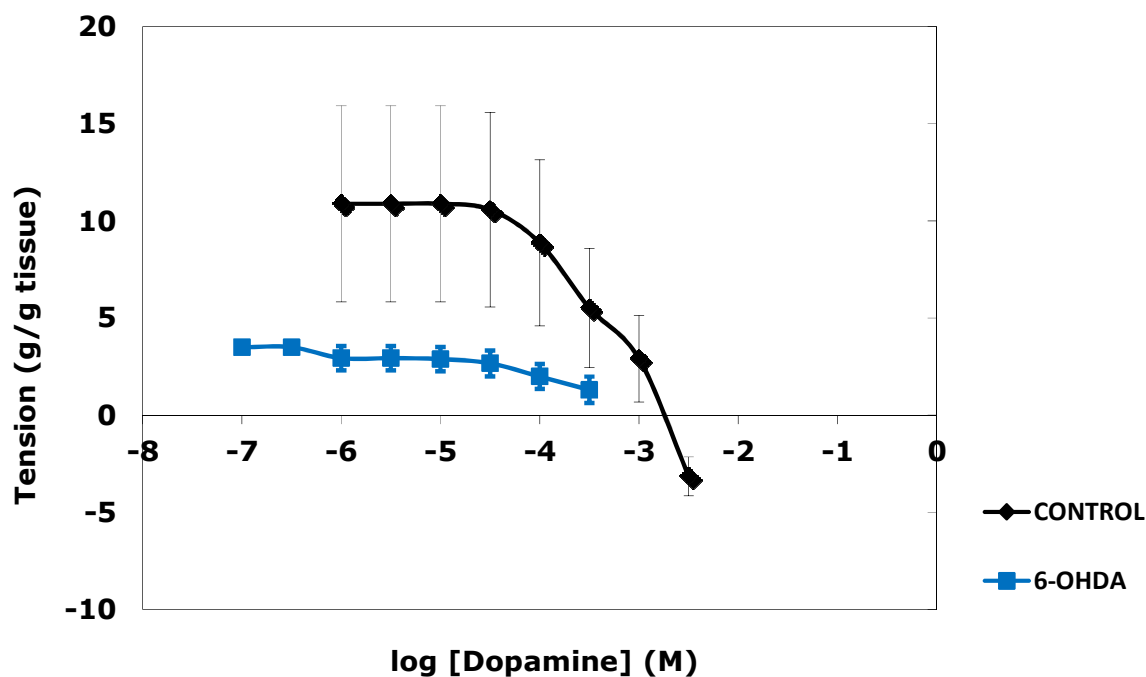


Fig. 55 Dose-response curve to Dopamine in the absence of nitrenergic contribution in control (n=7) and 6-OHDA rats (n=8) (circular strip of gastric fundus).

When the response to exogenous dopamine has been studied in the presence of D2 inhibitor Sulpride, it showed antagonism on both 6-OHDA and control tissues. It seems reasonable to conclude that the relaxant effect of dopamine is due to the activation of D2 receptors and there is no an important involvement of the nitrenergic component in the exogenous dopamine response.

Electrical stimulation

The tissue precontracted with PGF2 α has been subjected to electric field stimulation (EFS) with variable frequencies from 1, 2, 5 to 10 Hz (Fig. 56). The stimulus produced a frequency-dependent relaxation which in 6-OHDA tissues was almost half lower than control tissues: it is clearly evident how control tissues generally have a trend to relax more than 6-OHDA tissues.

In NANC conditions the EFS-induced relaxation both in 6-OHDA and control tissues is not modified compared to basal conditions.

The addition of L-NAME in NANC conditions abolishes the relaxant responses produced by stimulation at low frequencies of 6-OHDA tissues, giving a poor relaxing response to frequencies above 5 Hz (a fourfold lower relaxation than in NANC conditions). Also the control tissue responds with lower extent to the electrical stimulation in the presence of L-NAME (halved relaxation than

NANC conditions), within the entire range of frequencies applied. This data becomes statistically significant at 2 and 5 Hz in control tissues, at 1 and 2 Hz in 6-OHDA tissues (in the presence of α -chymotrypsin) (Fig. 56).

These observations indicate that mainly in 6-OHDA and in minor extent in control tissues there is a remarkable dependence of the relaxing response to NO release. This NO involvement is particularly evident at low frequency of electrical stimulation whereas at higher frequencies there is evidence of a residual relaxant response,. It is indeed well known that nitric oxide is released at low stimulation frequencies (Tonini et al., 2000).

The further application of α -chymotrypsin leaves almost unchanged the inhibitory responses to stimulation at high frequencies in the 6-OHDA tissues, while it seems to slightly strengthen them in the control tissues: this result indicates a possible involvement at high stimulation frequencies of peptidergic excitatory mediators in control tissues and not in 6-OHDA tissues.

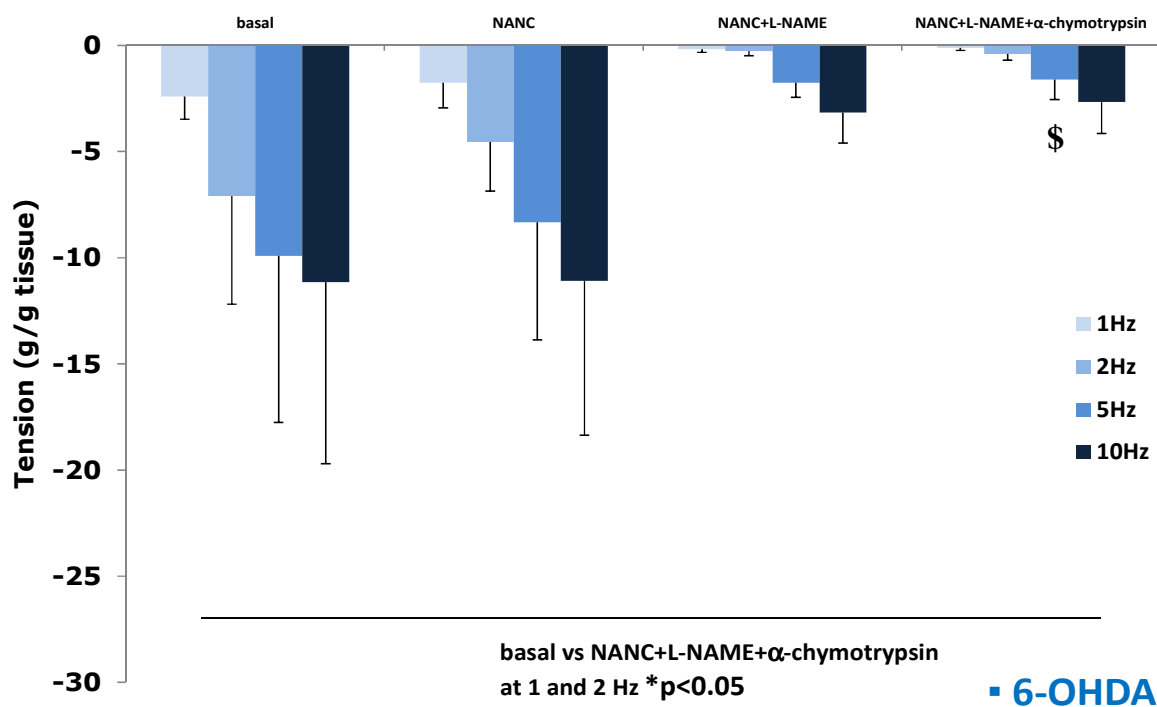
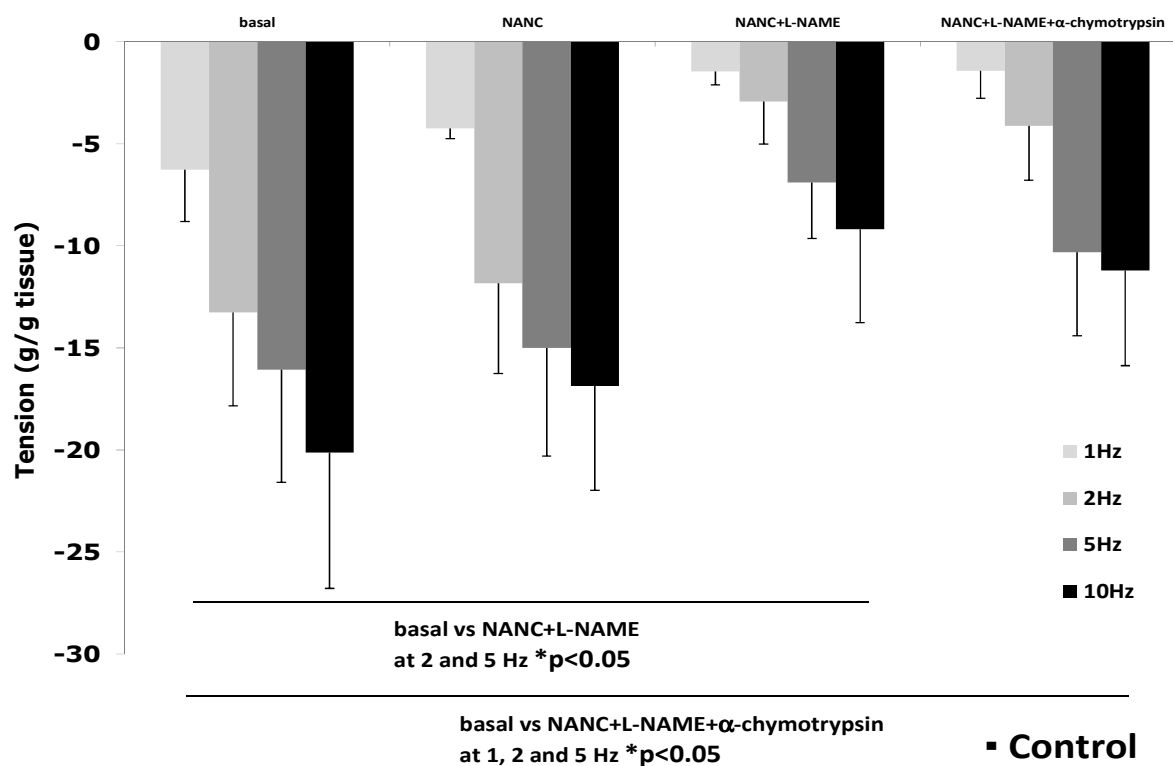


Fig. 56 Electrical field stimulation at 1, 2, 5 and 10 Hz in control (n=7) and 6-OHDA rats (n=8) (circular strip of gastric fundus). Student t-test \$ p<0.05 Control vs 6-OHDA.

Pylorus

Pharmacologic stimulation

• *Dose-response curve to bethanechol*

The pylorus arranged longitudinally and isolated from animals 6-OHDA responded to bethanechol with a contraction quite comparable to that shown by the tissue taken from the control animal (Fig. 57). The concentration-response curves obtained on the two types of tissues showed the same potency and efficacy:

Emax Control 9.45 g/g tissue - Emax 6-OHDA 9.48 g/g tissue
pD2 Control 4.72 - pD2 6-OHDA 4.75

It is therefore likely that, even after the lesion, the cholinergic muscarinic sensitivity in this district does not undergo alterations.

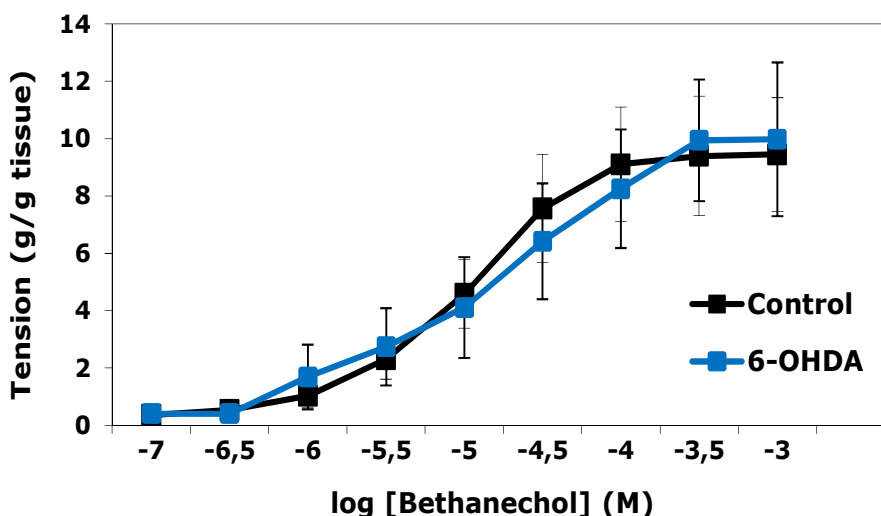


Fig. 57 Dose-response curve to bethanechol in control (n=7) and 6-OHDA rats (n=8) (pylorus).

• *Relaxation curves by Dopamine*

The application of increasing cumulative concentrations of dopamine on a hypertone produced by application of bethanechol (at concentrations corresponding to EC50 previously calculated) produces relaxation curve in the 6-OHDA tissue similar to what happens in the case of control animals. These data do not show significant differences between the tissues of the two different backgrounds (Fig.58): t-test [pD2 6-OHDA vs pD2 Control] p = 0.335 t-test [Emax 6-OHDA vs Emax Control] p= 0.288

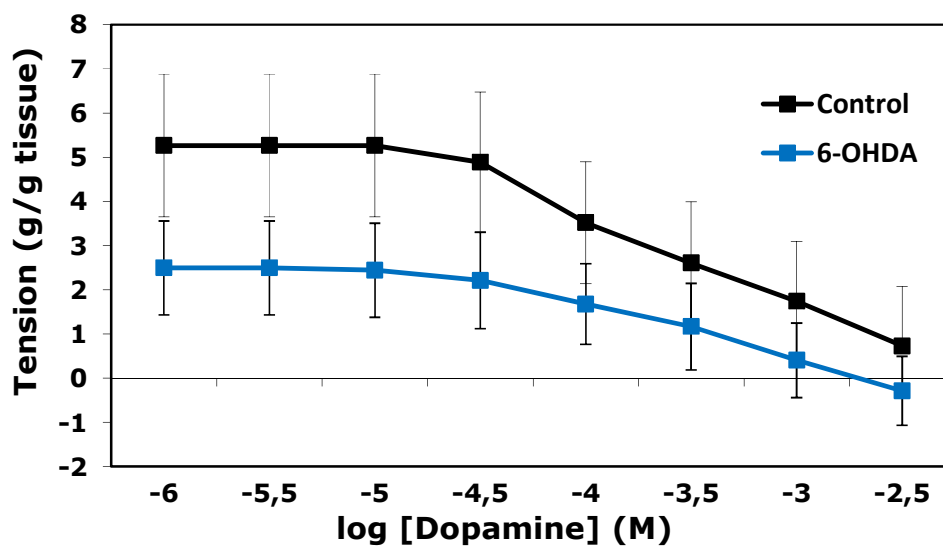


Fig. 58 Dose-response curve to Dopamine in control (n=7) and 6-OHDA rats (n=8) (pylorus).

It has been subsequently studied the tissue response to increasing concentrations of dopamine after incubation with Sulpiride 10⁻⁶ M (Fig. 59).

The inhibition of D2 receptors prevents the dopamine-induced relaxation of pyloric smooth muscle in control animals while it does not modify the response to dopamine in 6-OHDA tissues. At the level of the pylorus the D2-dependent inhibitory response seems therefore compromised, specifically, in 6-OHDA animals.

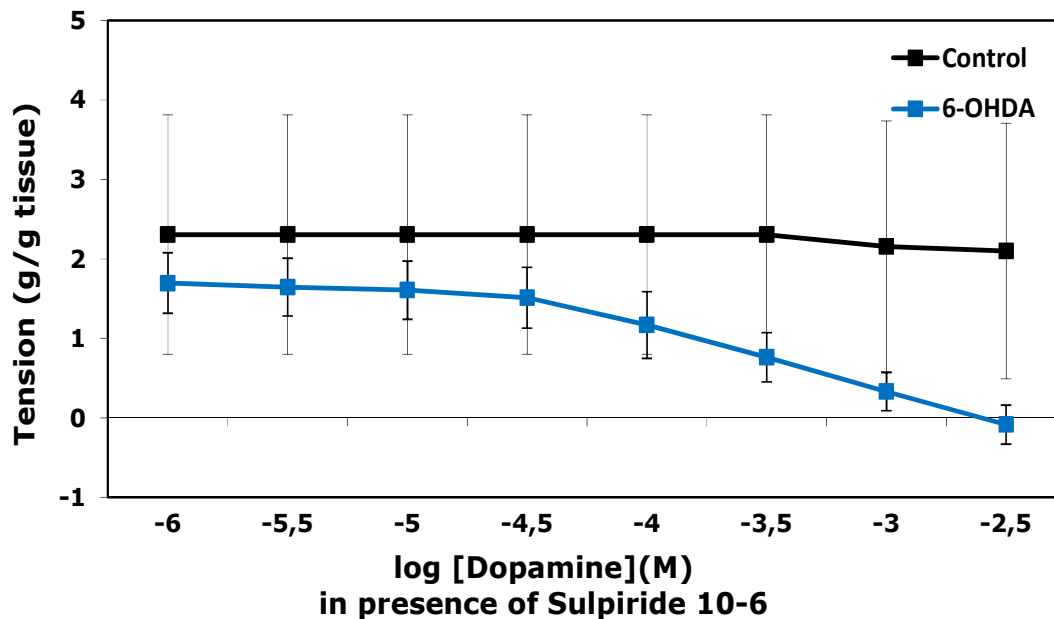


Fig. 59 Dose-response curve to Dopamine in presence of Sulpiride 10^{-6} M in control (n=7) and 6-OHDA rats (n=8) (pylorus).

• *Relaxation curves by dopamine in absence of nitrenergic and purinergic contribution*

Even in this case the relaxation induced by dopamine has been studied in the presence of 3×10^{-4} M L-NAME and 10^{-4} M Suramin (purinergic receptor antagonist). It did not show any statistically significant difference between the tissues in the two conditions (Fig. 60).

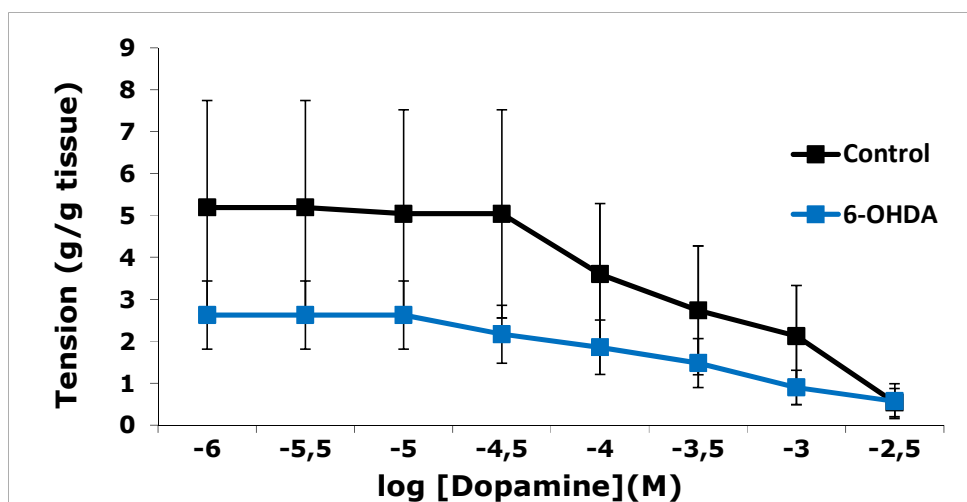


Fig. 60 Dose- relaxation curves by dopamine in presence of L-NAME and Suramin in control (n=7) and 6-OHDA rats (n=8) (pylorus).

Electrical stimulation

The control tissue at all frequencies tends to relax in a more pronounced extent than 6-OHDA tissue (about 4-5 g/g tissue of control vs 2.5 g/g tissue of 6-OHDA in Figure 61), although the statistical analysis did not report any relevant significance.

It is well known that, together with the cells of Cajal (ICC), the nitrenergic innervation can account for the relaxation of the pylorus (Sivarao et al., 2008): indeed, in the presence of L-NAME, an appreciable decrease of relaxation in Control tissues was observed. On the contrary no changes in neurogenic relaxation was displayed by 6-OHDA tissues, suggesting an almost total loss of nitrenergic control of pyloric junction motility after central dopaminergic deficit. The combination of L-NAME + Suramin had no additional effects, suggesting that it may be excluded a purinergic role in this type of motor response.

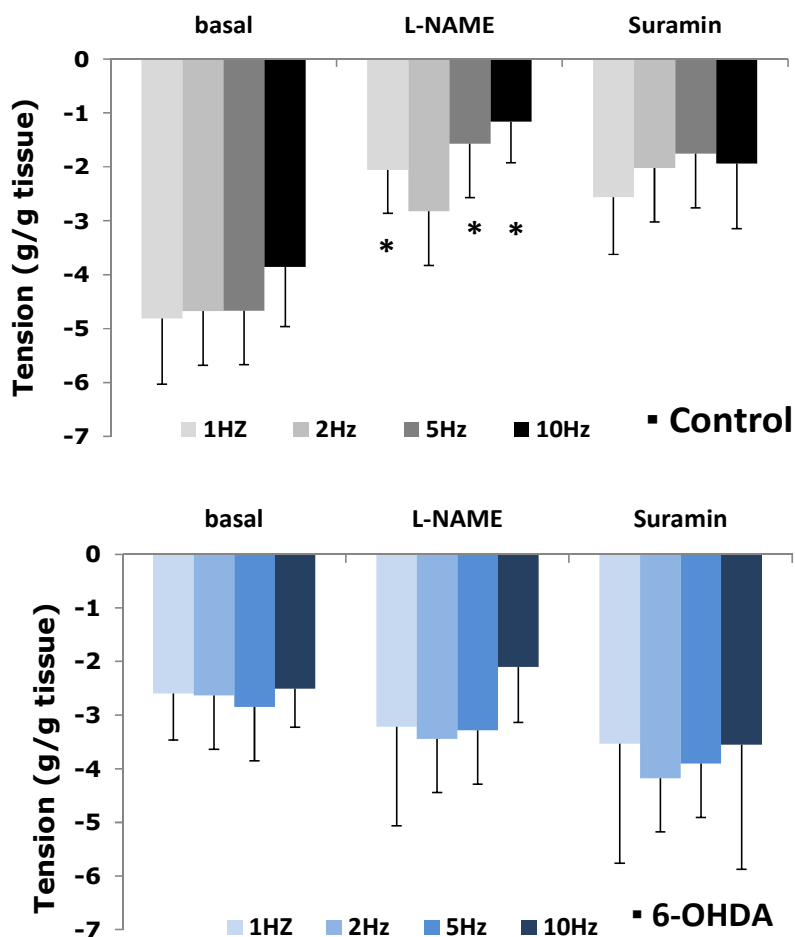


Fig. 61 Electrical field stimulation at 1, 2, 5, 10 Hz in control (n=7) and 6-OHDA rats (n=8)
*p<0.05 basal vs L-NAME in control animals (pylorus).

Longitudinal gastric strip

Pharmacological stimulation

• Dose-response curves to bethanechol

Evaluating the effect of bethanechol on the gastric longitudinal strip and comparing the data obtained in control animals with those obtained in 6-OHDA rats, it has been noticed a comparable behaviour (Fig. 62).

Emax Control 11.9 g/g tissue - Emax 6-OHDA 9.3 g/g tissue [t-test $p=0.487$]

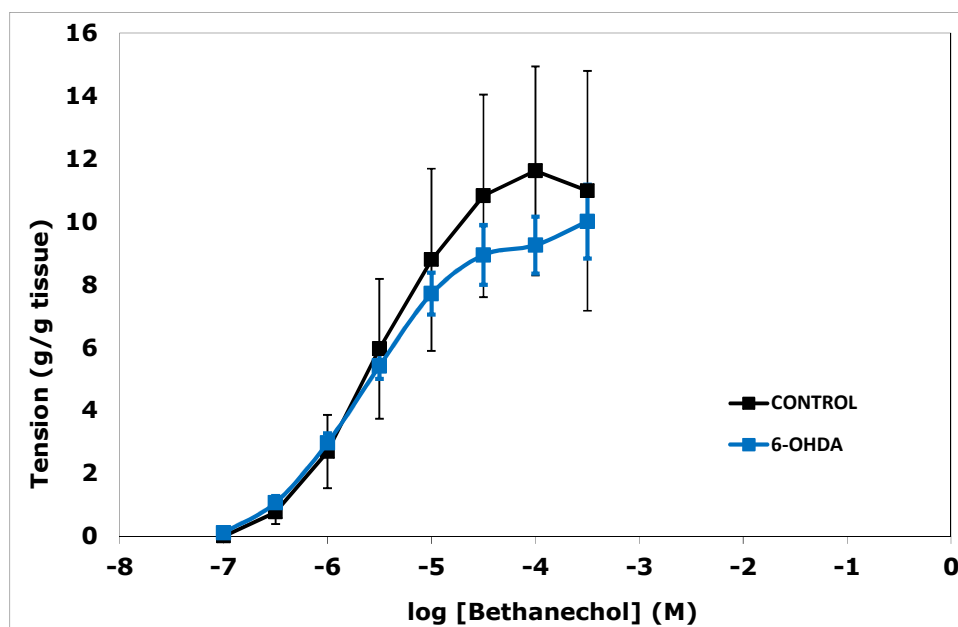


Fig. 62 Dose-response curve to bethanechol in control (n=7) and 6-OHDA rats (n=8) (longitudinal strip of stomach).

• Dose-response curve by $PGF2\alpha$

The graph shows a significant higher potency of $PGF2\alpha$ in 6-OHDA tissues compared to controls (Fig. 63). It should however be noted, in control tissue, a generally high standard error; the difference is not significant in statistical terms.

Emax Control 4.69 g/g tissue - Emax 6-OHDA 4.88 g/g tissue [t-test = 0.760]

PD2 Control 6.79 – PD2 6-OHDA 7.60 [t-test = 0.014]

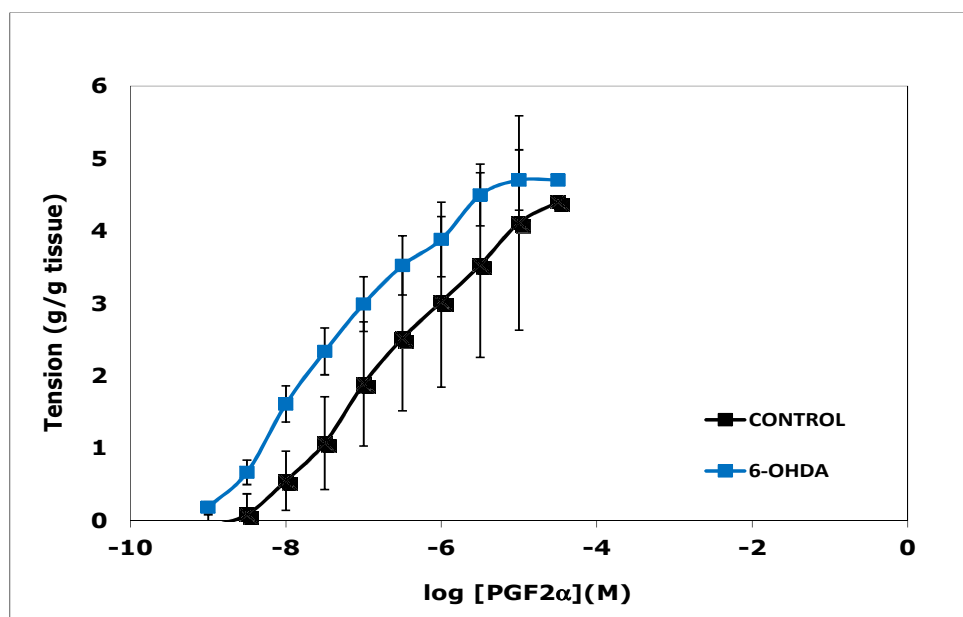


Fig. 63 Dose-response curve to PGF2 α in control (n=7) and 6-OHDA rats (n=8) (longitudinal strip of stomach).

• *Relaxation curves by dopamine*

Even in this case, in response to increasing concentrations of dopamine, the 6-OHDA tissue (precontracted with a concentration of PGF2 α equal to its EC50) shows a lower relaxation compared to control tissue (although there is no appreciable statistical significance: t- test [pD2 Control vs pD2 6-OHDA] p= 0.088); It can be noted that both reach lower values compared to the baseline condition (Fig. 64).

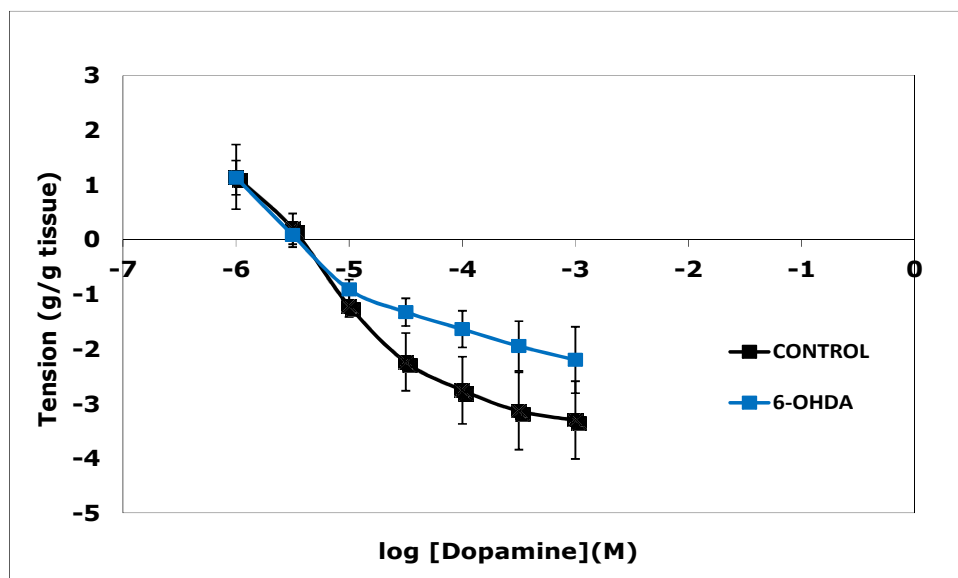


Fig. 64 Dose-response curve to Dopamine in control (n=7) and 6-OHDA rats (n=8) (longitudinal strip of stomach).

The relaxation responses produced by dopamine were antagonized by Sulpiride 10^{-6} M in the two types of tissues: the control tissues appear to be more susceptible to the D2-blockade since the reduction of dopamine efficacy and potency in 6-OHDA tissues was less marked than in control ones (Emax Control and 6-OHDA in the absence of antagonist = 100.00% - Emax Control in the presence of antagonist = 29.40% / Emax 6-OHDA in the presence of antagonist = 79.15%) (Fig. 65). Thus, in agreement with the observations made previously in the pylorus, also the longitudinal strip seems to display an impaired inhibitory D2-dependent control in 6-OHDA animals.

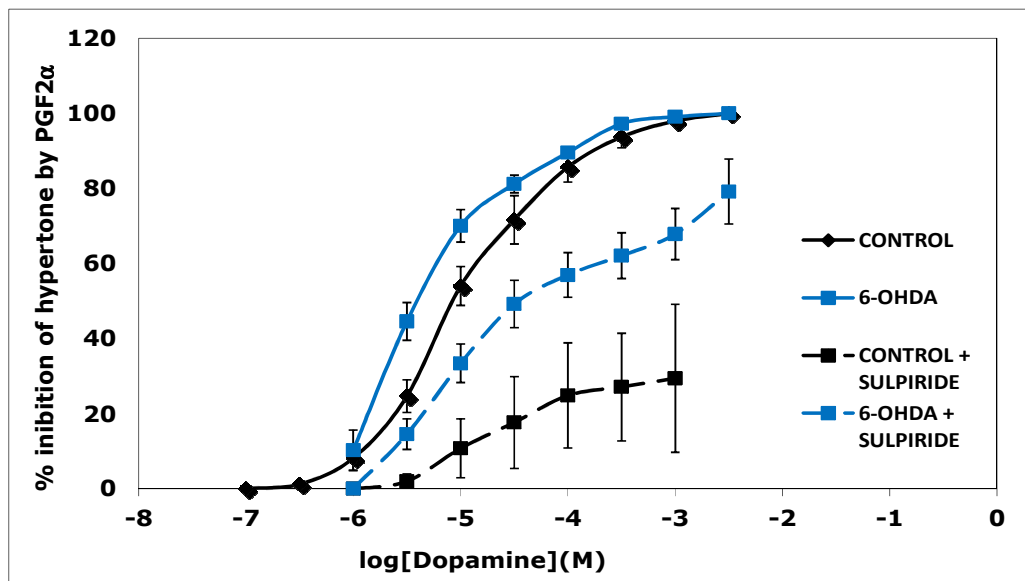


Fig. 65 Percentage of inhibition of the $\text{PGF}_2\alpha$ -induced hypertone by Dopamine in the presence of Sulpiride in control (n=7) and 6-OHDA rats (n=8) (longitudinal strip of stomach).

• *Relaxation curves by dopamine in the absence of nitrenergic contribution*

Repeating the same experiment after incubation with L-NAME, 6-OHDA tissues showed superimposable curves with control tissues (Fig. 66). These results indicate that in 6-OHDA tissues dopamine induces a relaxing effect partially dependent on the release of endogenous NO. This original result does not find support in the literature since studies in this field have not yet been carried out in-depth.

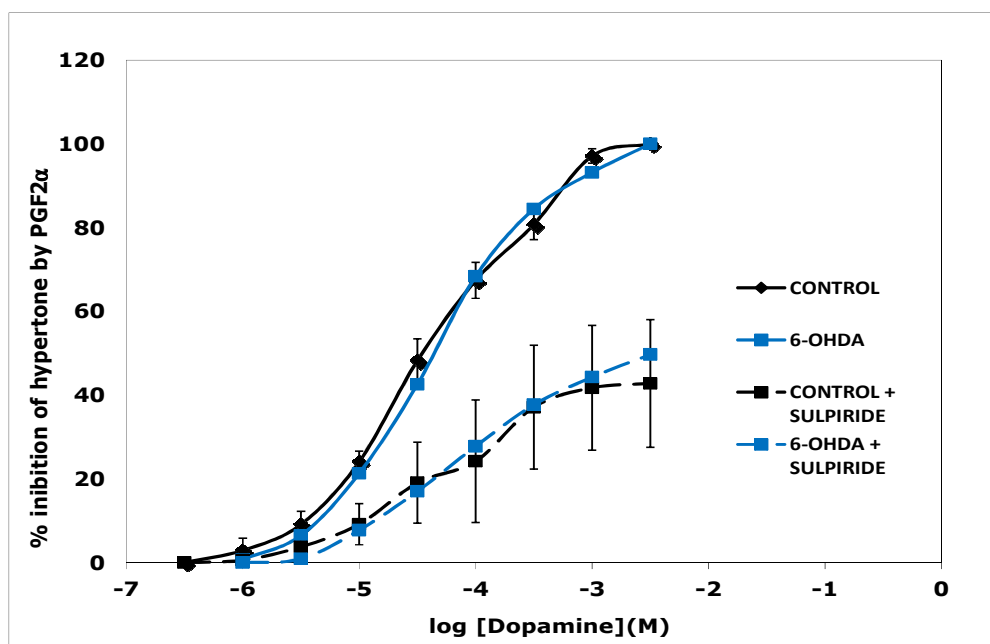


Fig. 66 Dose-response curve to Dopamine in absence of nitrenergic contribution in control (n=7) and 6-OHDA rats (n=8) (longitudinal strip of stomach)

Electrical stimulation

Electrical stimulation from 1 to 10 Hz causes in basal conditions a slight relaxation of the control tissues while there is almost no effect in 6-OHDA tissues (see graphs in Figure 67). The NANC conditions reveal a relaxation response higher in the control tissues compared to 6-OHDA but without reaching a statistical significance like in the second. The application of L-NAME halved the relaxing response in the two types of tissues (*p<0.05 in control, **p<0.05 in 6-OHDA tissues). The application of the protease α -chymotrypsin maintained almost unchanged the inhibitory response to EFS presented by both tissues 6-OHDA and control.

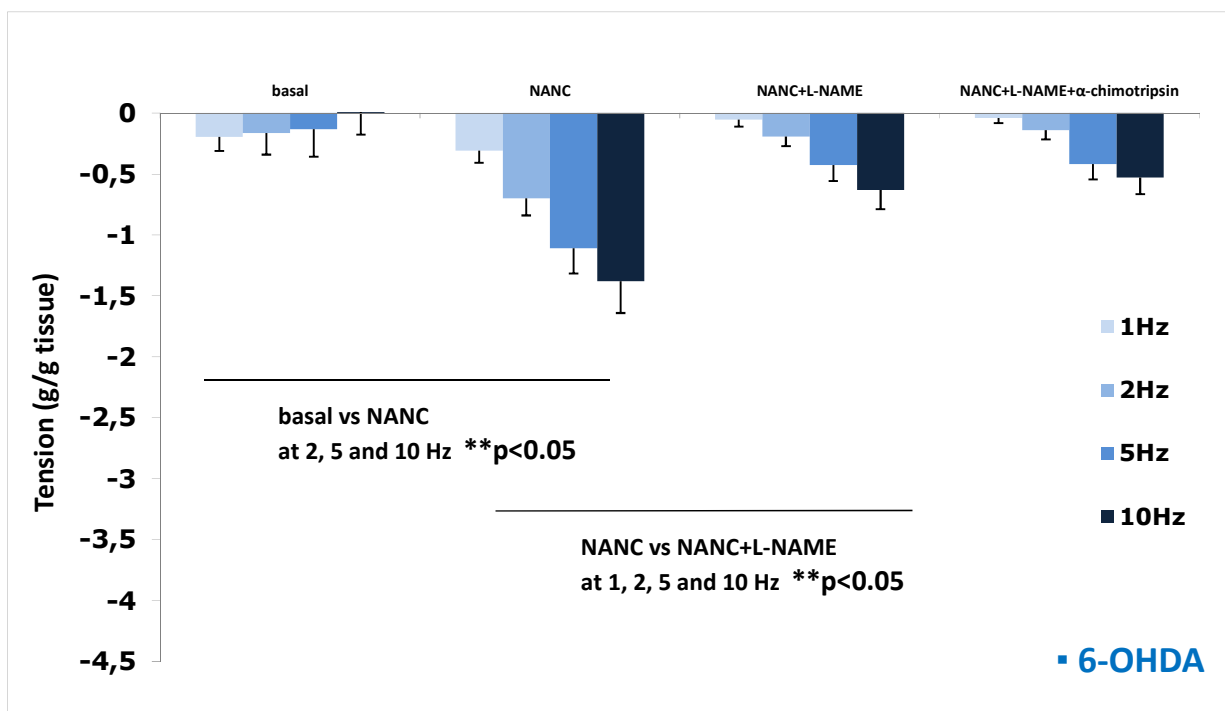
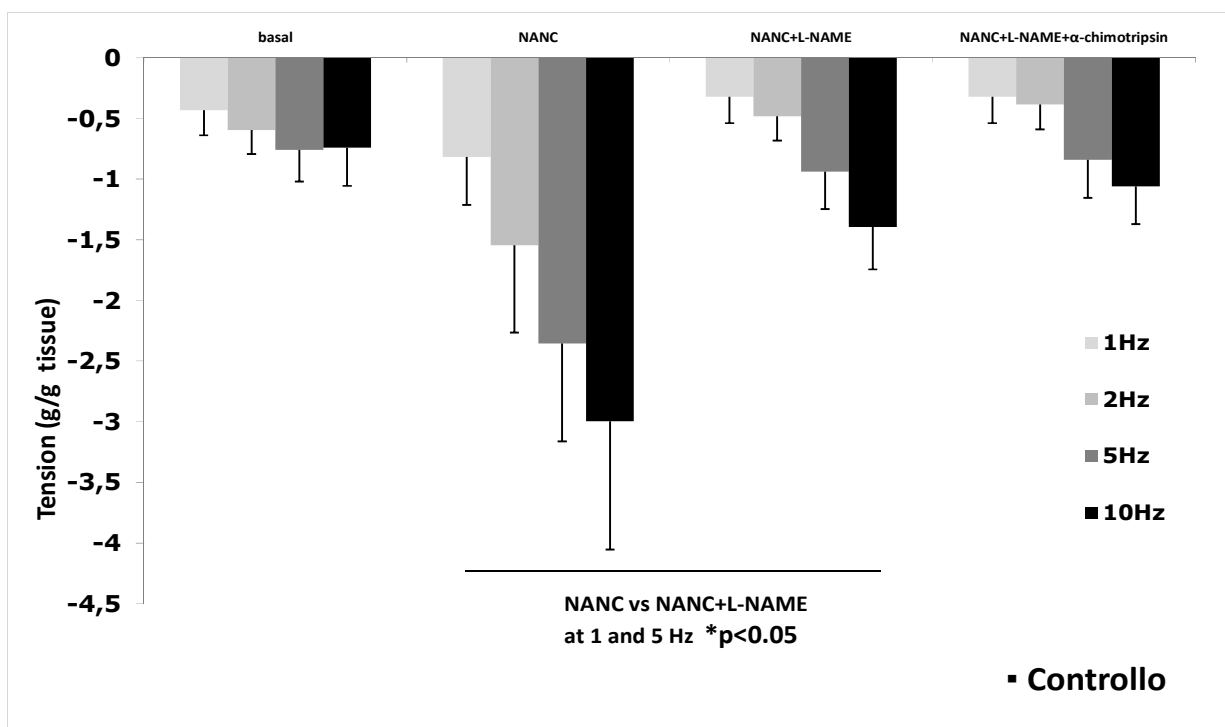


Fig. 67 Electrical field stimulation at 1, 2, 5 and 10 Hz in control (n=7) and 6-OHDA rats (n=8) (longitudinal strip of stomach).

Malondialdehyde (MDA) assay

To investigate the possible mechanisms underlying the alterations in gastrointestinal motility, it has been quantified a marker of oxidative stress and in particular the marker of lipoperoxidation: Malondialdehyde (MDA). This assay, performed at the eighth week after the central lesion, showed that the tissue samples taken from 6-OHDA rats tend to display a higher degree of oxidative stress compared to control rats. In particular, the values of MDA in the samples of the stomach fundus and corpus, jejunum and ileum assume statistical significance ($*p<0.05$) indicating a particular increase in oxidative stress in these segments. Also in distal colon of 6-OHDA rats MDA values were almost doubled compared to control but the high variability precludes obtaining statistical significance.

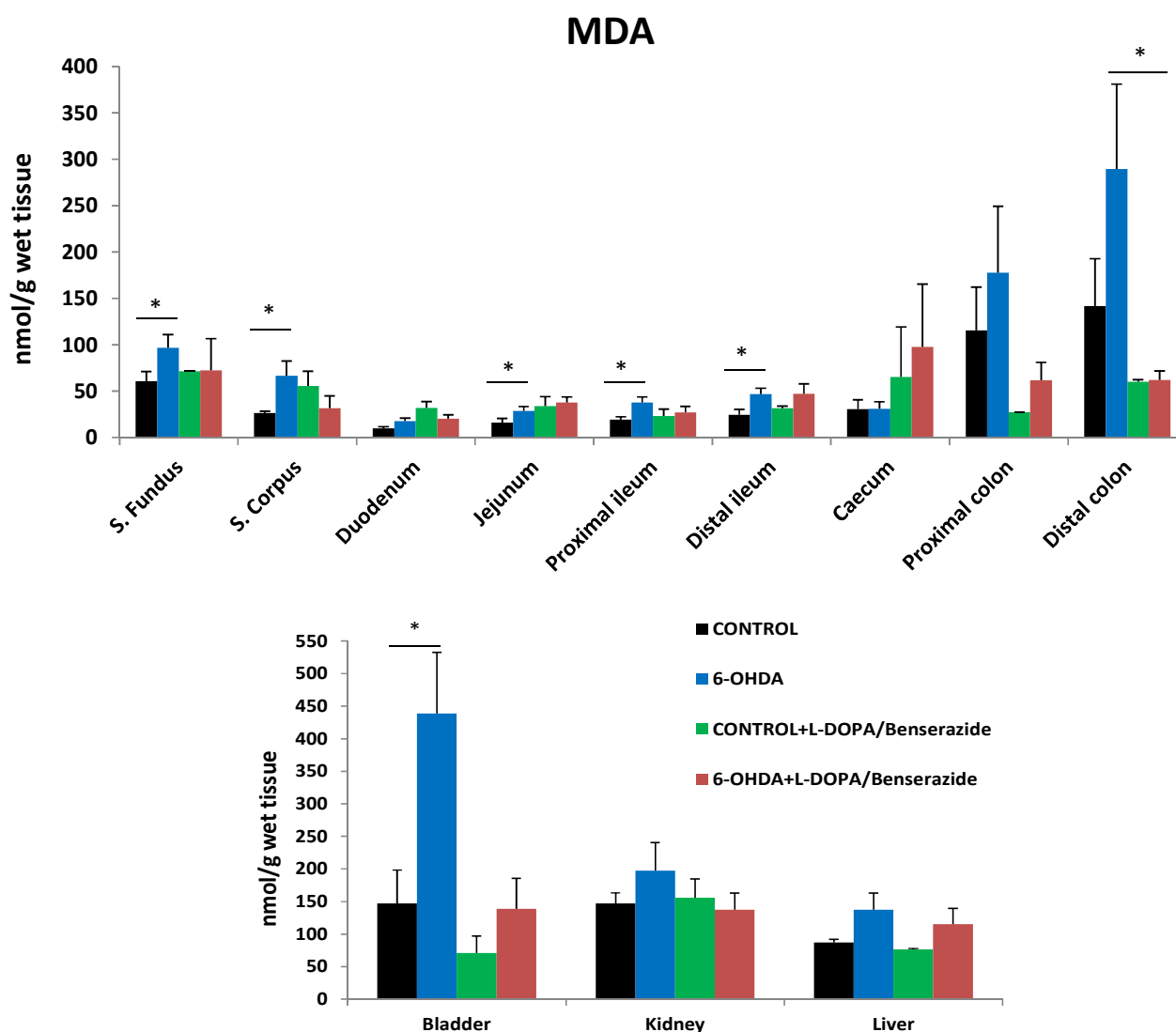
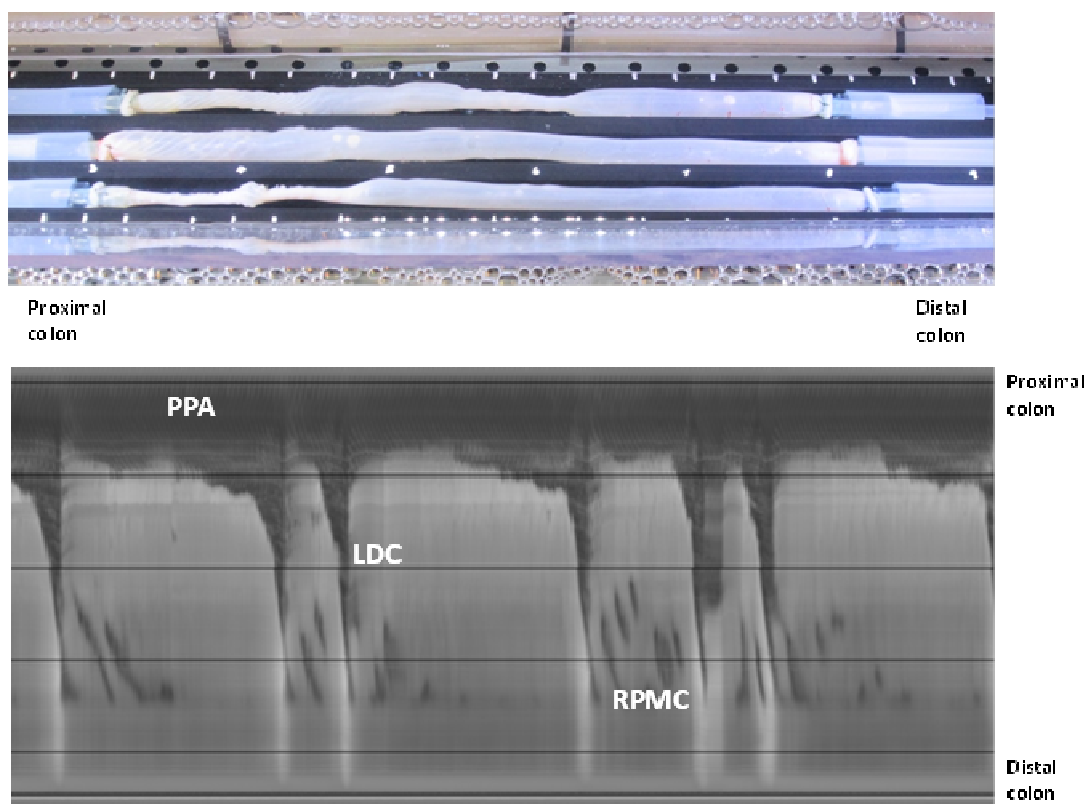


Fig. 68 MDA levels in different GI districts and other organs in control (n=6), 6-OHDA (n=12), control/ treated (n=2) and 6-OHDA/ treated rats (n=5).

As it can be observed from Figure 68, the subchronic treatment with L-DOPA/Benserazide tends to reduce the condition of oxidative stress affecting the stomach corpus and the proximal colon even if it does not reach statistical significance, with the exception of the distal colon where the difference is clearly significant $*p < 0.05$ 6-OHDA vs 6-OHDA/treated rats.

Part 3A: Study of the rhythmic pan-colonic propulsive motor patterns and their spatiotemporal organization in control and PD rats.

After 4 weeks from surgery there are no relevant changes between controls and 6-OHDA colon in terms of *long distance contractions* (LDCs) and *rhythmic propulsive motor complexes* (RPMC) following incubation of D2R antagonist L-741626 10^{-6} M. On the contrary, at 8 weeks the colon of control animals responds normally with an increase in propulsive contractions, showing an intact intrinsic dopaminergic activity, while that of 6-OHDA animals exhibits a negligible response. This suggests the lack of expression of D2 receptors in the colon or their inactivation.



The following figures represent the spatiotemporal maps obtained from video recordings of rat colon motility (showing the motor patterns) of control and 6-OHDA rats after 8 weeks from central lesion.

The figures 69a and 69b (recordings lasting 20 min) show the **LDCs** and **RPMCs**:

-During the period of stabilization or baseline in the control (1) and 6-OHDA (3)

-After addition of D₂R antagonist L-741626 10^{-6} in the control (2) and 6-OHDA (4)

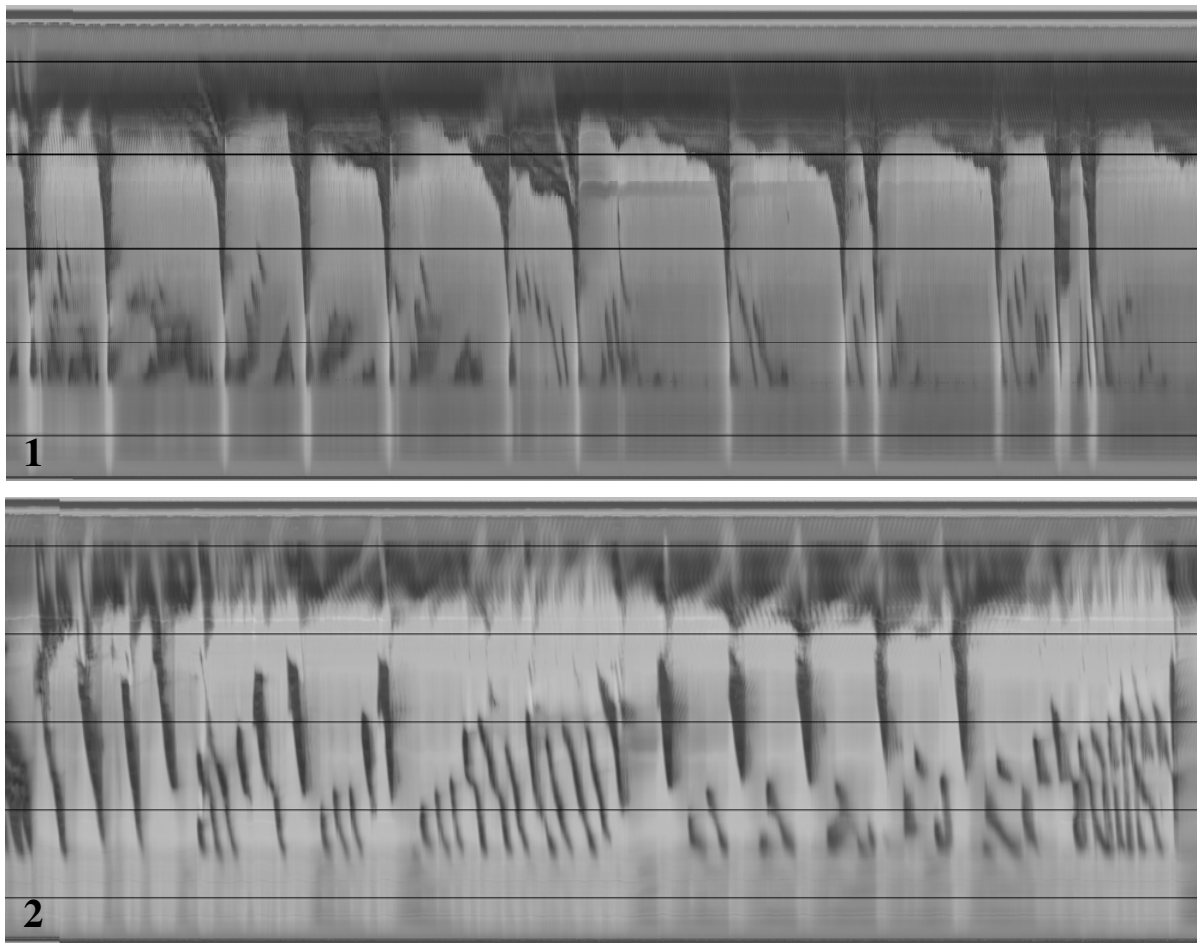


Fig. 69a Spatiotemporal maps created from video recordings of motor patterns of the whole control rat colon (n=5). The figures show LDCs and RPMCs during the period of stabilization (panel 1) and immediately after the addition of D₂R antagonist L-741626 10^{-6} (panel 2).

All the panels shown are representations of 20 min recordings.

In the presence of the D₂R antagonist, the regular Long Distance Contractions (LDCs) lose their typical aspect with large contractile activity in the proximal colon and thin relaxation in the distal colon, becoming instead more frequent and short pan colonic LDC-like activity. This is what expected from the D₂ receptor antagonist whose property is to increase the spontaneous contractile activity. On the other hand this drug is able to generate also more Rythmic Propulsive Motor Complexes (RPMCs) which are responsible for the onset of the segmentation activity, characteristic of the distal colon peristalsis.

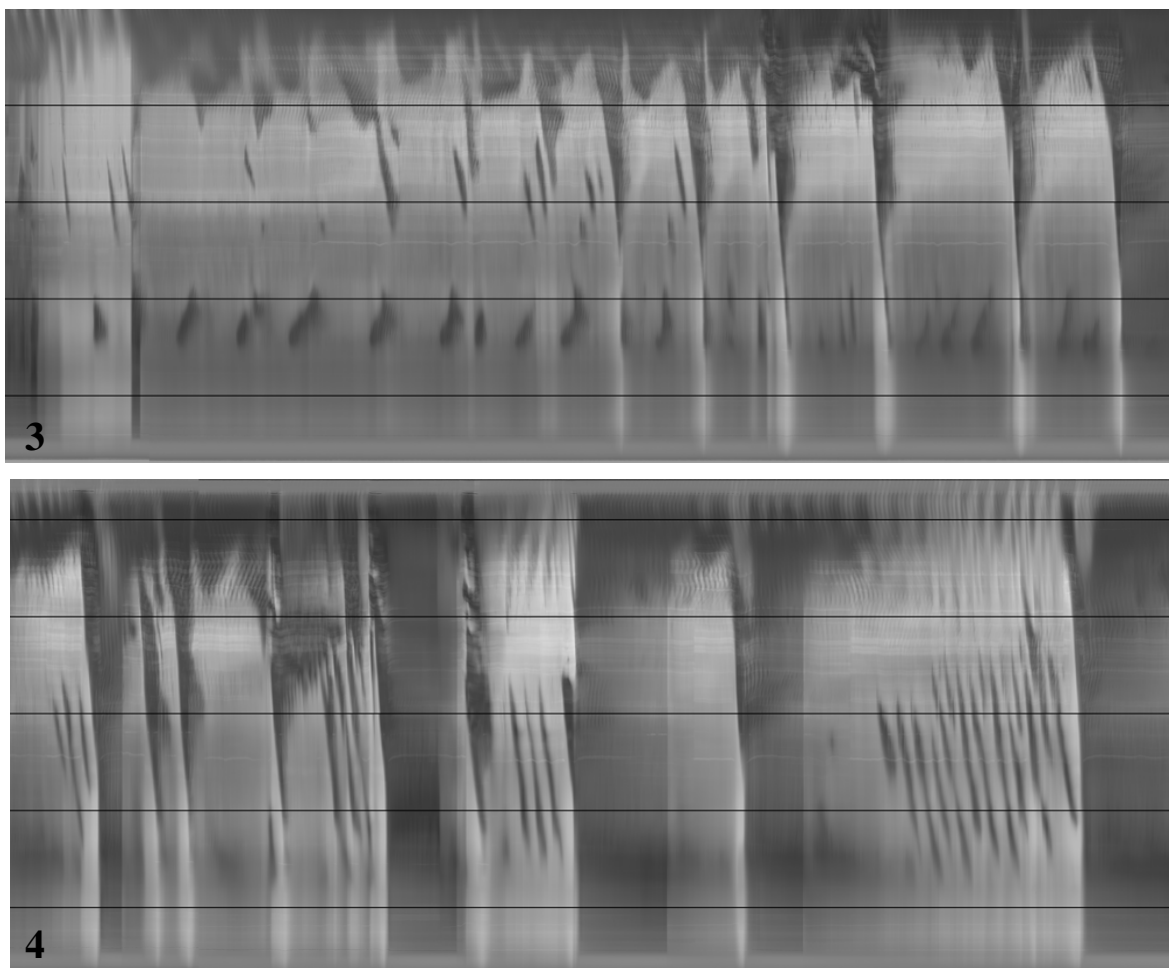


Fig. 69b Spatiotemporal maps created from video recordings of motor patterns of the whole 6-OHDA rat colon (n=5). The figures show LDCs and RPMCs during the period of stabilization (panel 1) and immediately after the addition of D₂R antagonist L-741626 10⁻⁶ (panel 2). All the panel shown are 20 min recordings.

In basal condition 6-OHDA rat colon exhibits a less intense spontaneous motor activity compared to control colon. The addition of the D₂R antagonist does not show the same consequent increasing of contractile activity as in control rat colon. There are no LDCs, but some irregular contractions and a few RPMCs with an elongated shape only in the mid colon.

The spatiotemporal maps can be assessed in many different ways, in a qualitative way describing the aspect and shape of the motor patterns, their length, width, % of relaxation (white color) but also in a preliminary quantitative way by calculating motor pattern frequency in a definite time interval. So here what it can be reported is that control rat colon presents an RPMCs frequency of 0.55/min during stabilization and 1.33/min after the addition of the D₂R antagonist. On the contrary the 6-OHDA rat colon has unchanged frequencies before and after the addition of the drug: RPMCs frequency of 0.55/min during stabilization and 0.5/min after D₂R antagonist addition. The analysis of

the two motor patterns is semiquantitative and limited just to the evaluation of their frequency. It is noteworthy that the semiquantitative partial parameter does not describe completely the motility profile.

Part 3B: Detection and quantification of Interstitial Cells of Cajal (ICC) and nitrergic nerves in the stomach and colon

The immunohistochemical studies showed interestingly a significant increase of ICC density in some regions of the stomach in the 6-OHDA animals 4 and 8 weeks after surgery. This is accompanied by a similar significant increase of n-NOS expression in the same regions. From many extensive studies on the ICCs we know that they have a particular plasticity, with an ongoing loss and replacement in few days or weeks (Farrugia G. 2008). The phenomenon of oxidative stress can be the major cause of their early damage and loss but at the same time can induce an important pro inflammatory response mediated by the mastcells through the production of mediators like interleukines like Il-4, Il-6 and Il-9. Particularly Il-9 has been demonstrated in literature to have a role as a growth factor and a stimulus of regeneration of the ICCs, as well as SCF (stem cell factor), 5HT (serotonin), PDGF and others. Oxidative stress and inflammation cause damage but parallel do arise reparation mechanisms. So probably the endpoint of the experiments corresponds to a moment of peak of ICCs proliferation after damage.

Regarding the parallel increase of n-NOS expression, it is noteworthy that ICCs and nitrergic neurons are structurally and functionally interconnected since they are located in proximity and neuronally derived nitric oxide is a molecule that can induce ICCs to proliferate (Farrugia G. 2008).

The c-kit-positive area percentage or the ICC density in 6-OHDA rats are similar in the 3 parts of the stomach (fundus 1.2%, corpus 0.98% and antrum 1.3%) 4 weeks after surgery. While it decreases in the corpus 0.5% and antrum 0.68%, remaining the same in the fundus 0,93% 8 weeks after surgery, comparing to controls (Fundus 0.31%, Corpus 0.41%, Antrum 0.31%). The values are almost halved and the differences are statistically significant (Fig. 70).

Although the 8 weeks values remained in both endpoints higher than the controls, they are lower than the 4 weeks values.

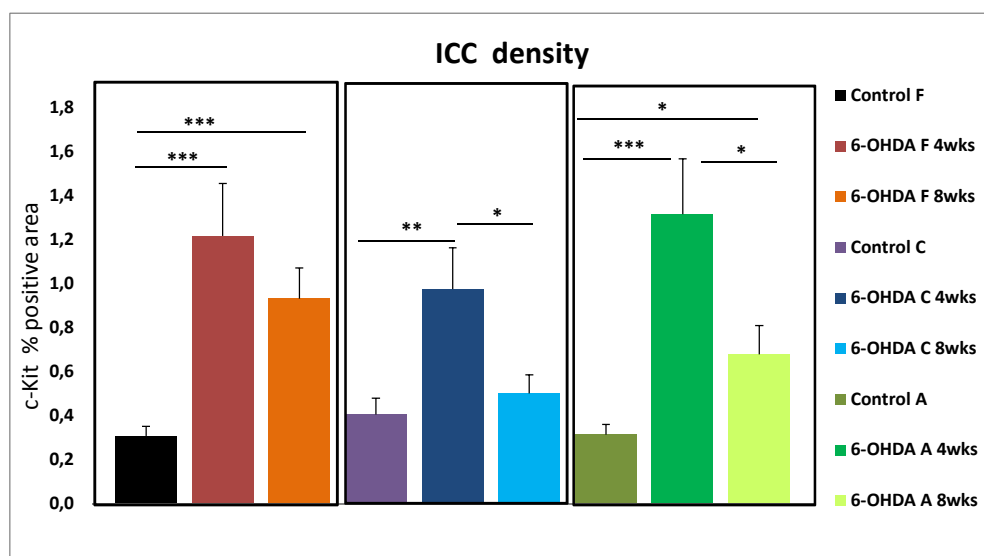


Fig. 70 Percentages of c-Kit positive area in stomach fundus, corpus and antrum in 6-OHDA (n=5) and control rats (n=5) after 4 and 8 weeks from surgery.

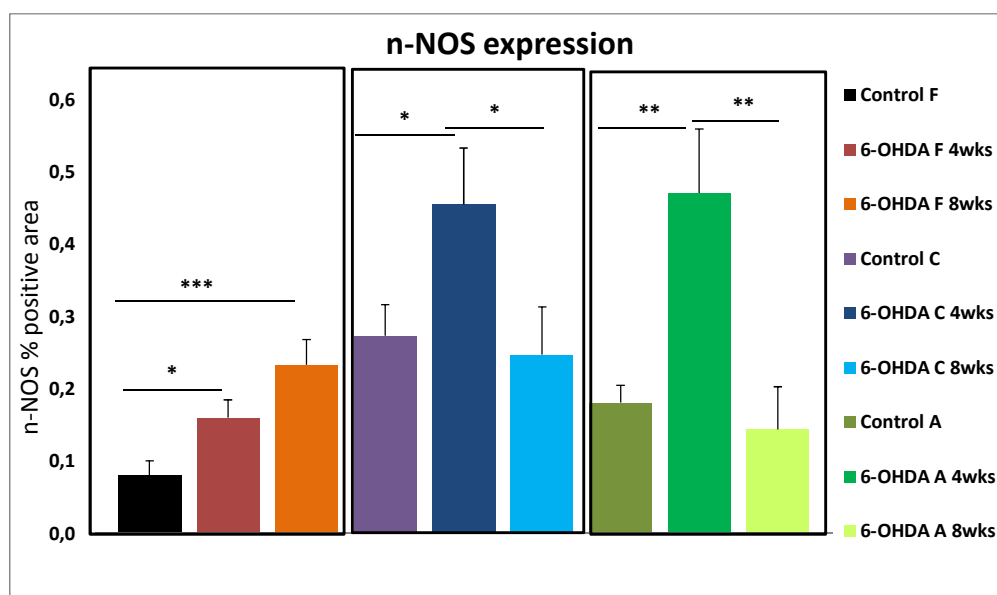


Fig. 71 Percentages of n-NOS positive area in stomach fundus, corpus and antrum in 6-OHDA (n=5) and control rats (n=5).

The n-NOS positive area percentage or simply the n-NOS expression in 6-OHDA tissues is more pronounced in the stomach corpus and antrum than the fundus (fundus 0.16%, corpus 0.46% e antrum 0.47%) 4 weeks after lesion. While after 8 weeks it becomes in the fundus 0.23%, corpus 0.25% and in antrum 0.14%. Even here, although the 8 weeks values remained in both endpoints higher than the controls, they are lower than the 4 weeks values in stomach corpus and antrum (Fig. 71) but not in the fundus where the % positive area is more than doubled with respect to control.

The c-kit-positive area percentage or the ICC density in 6-OHDA rats doesn't change in the colon comparing to the control after both 4 and 8 weeks. While the NOS positive area density is increased after 4 and 8 weeks from surgery in the distal colon (Fig. 72).

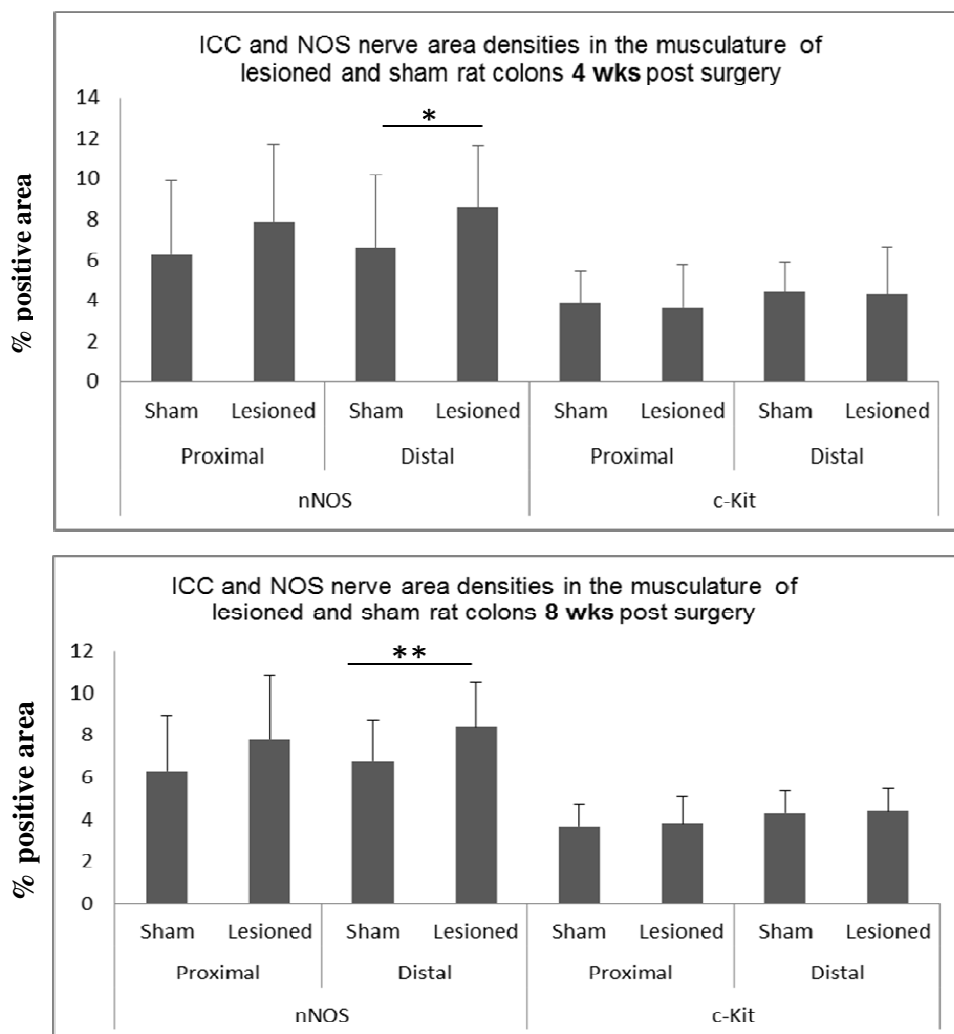


Fig. 72 Percentages of c-Kit and n-NOS positive areas in the colon of 6-OHDA (n=5) and control rats (n=5) after 4 and 8 weeks from surgery.

So, the changes in n-NOS expression and ICC density seem to follow a similar temporal trend relatively to the nigrostriatal lesion.

The mastcells detected by the confocal microscope on whole mount preparations and their number identified through counting on frozen section preparations showed important conclusions:

- 1- They increase only in Fundus and Antrum and not in Corpus of the stomach in 6-OHDA rats 4 weeks after lesion.
- 2- They decrease in the same regions mentioned above returning to control values at 8 weeks.

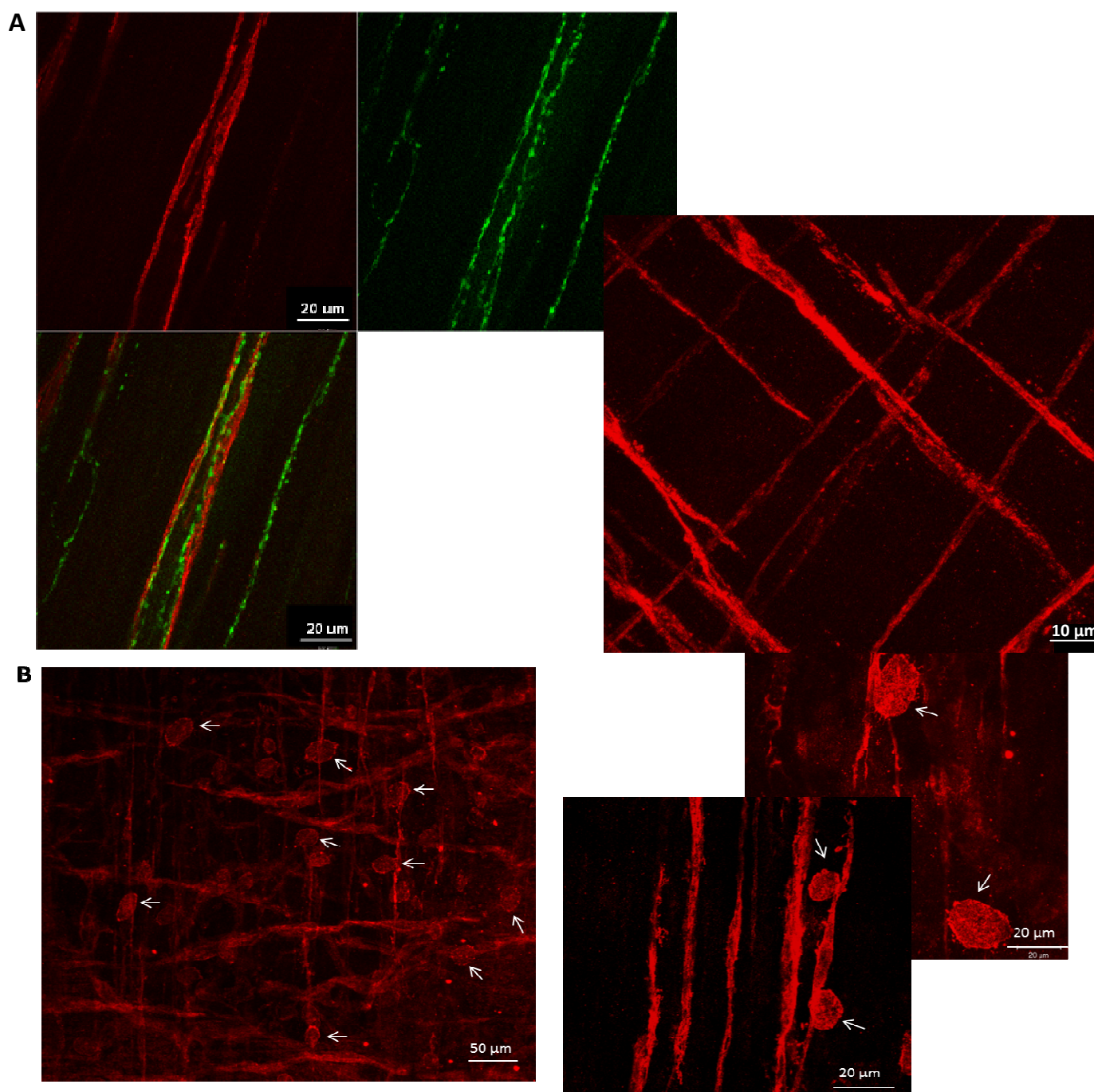


Fig. 73 c-Kit positive ICC-IM (red) and NOS positive nerves (green) in stomach of control (A) and 6-OHDA (B) rats. c-Kit antibody stains ICC and n-NOS antibody stains nitergic fibers. Top figures: NOS positive nerves (green) run parallel to and intimately associate with several ICC-IM (red) in the circular muscle layer. Bottom figures: mastcells (indicated by arrows) in close contact with ICC-IM.

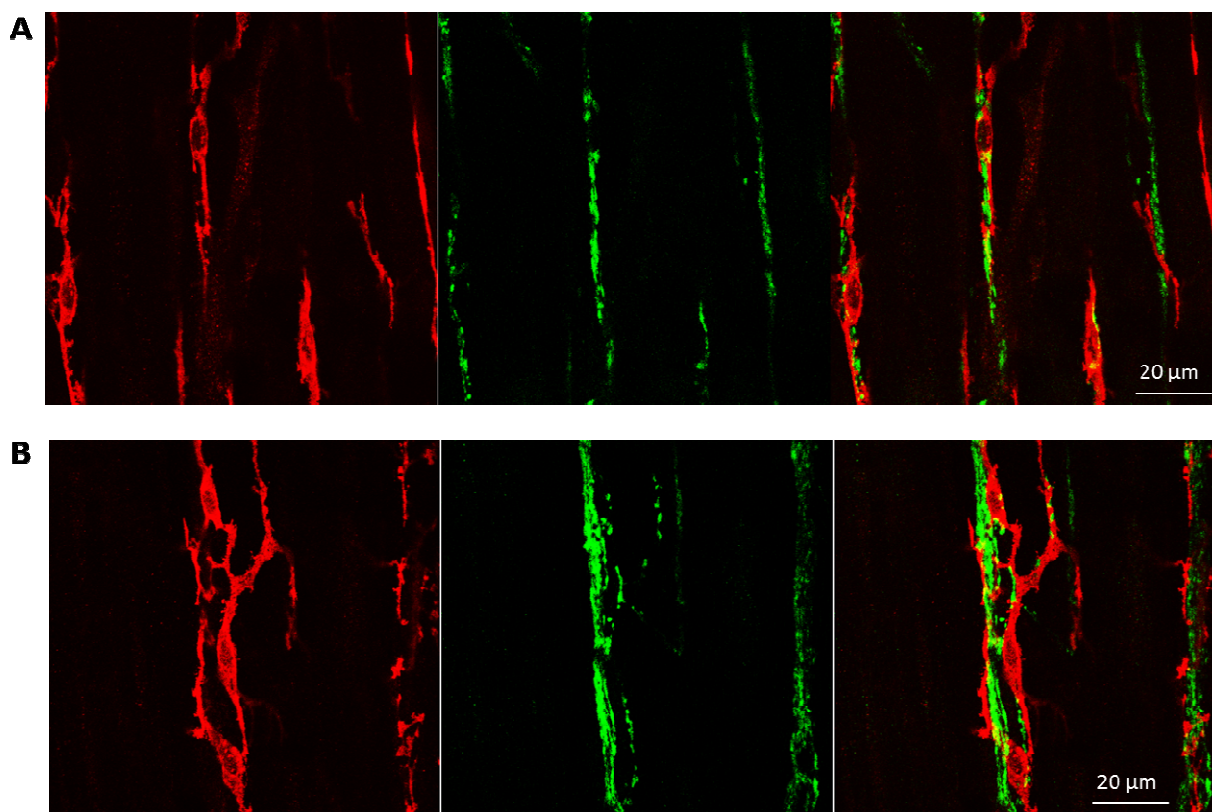


Fig. 74 c-Kit and n-NOS positive area in colon of control (A) and 6-OHDA (B) rats. c-Kit antibody stains ICC and n-NOS antibody stains nitrergic fibers.
 c-Kit positive ICC-IM (red) and NOS positive nerves (green) in colon of control (A) and 6-OHDA (B) rats. c-Kit antibody stains ICC and n-NOS antibody stains nitrergic fibers.
 NOS positive nerves (green) run parallel to and intimately associate with several ICC-IM (red) in the circular muscle layer (third panel on the right).

Discussion and conclusions

Part 1: Consequences of an acute nigrostriatal damage on the peripheral non-motor functions in rats, especially at the level of the digestive tract.

The purpose of this first part of the research was to verify if the damage of the nigrostriatal dopaminergic system, condition that characterizes Parkinson's disease, may be associated with alterations in non motor systems, with particular regard to the gastrointestinal (GI) system.

The aetiology of Parkinson's disease, aging-related disease characterized by progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta, is not yet fully clarified. The typical motor symptoms, consisting in extrapyramidal dysfunctions, are often associated with non-motor symptoms involving the gastrointestinal tract, such as constipation and disorders related to alterations in gastric emptying. These events degrade the quality of life of the patient and may interfere, as in the case of changes in the normal intestinal peristalsis, with the proper absorption of drugs used in the treatment of the disease itself. Moreover they can appear in very early stage of the disease, or even before the onset of motor symptoms, until they have been mentioned as possible pre-clinical signs (Yuncheng Wu et al., 2011).

In order to prove the involvement of the enteric nervous system in the progression of Parkinson's disease, Wakabayashi et al. (1989) demonstrated the presence of cytoplasmic inclusions identifiable with Lewy bodies in the myenteric plexus of patients suffering from the disease. But despite this, it still remains unclear whether the gastrointestinal dysmotility is due to primary alterations of central or peripheral dopaminergic circuits.

The hypothesis of a connection between dopaminergic damage and gastrointestinal motility dysfunction has been investigated through studies using animal models. For example it has been highlighted an altered intestinal myoelectrical activity after duodenal administration of MPTP in mice and non-human primates (Szabo et al., 1990). This toxin, administered intraperitoneally to mice causes very rapidly a selective decrease of dopaminergic neurons in the ENS (40%), without numerical

alteration of cholinergic and nitrergic neurons (Anderson et al., 2007). Indeed, the alterations of the gastro-intestinal motility associated with the administration of MPTP were discordant. It appears that colonic motility undergoes a transient acceleration in the MPTP model in mice (Anderson et al., 2007), unlike what happens in the model by 6-OHDA in which the destruction of central nigrostriatal dopaminergic pathways is associated with the development of constipation (Blandini et al., 2009; Colucci et al., 2012).

Concerning gastric emptying, which is often slowed in patients (Hardoff et al., 2001), there are conflicting data in literature. Evidence of a delay of transit toward the intestine is found both in the MPTP model in mice, in the 6-OHDA (Zhu et al., 2012) and also in the rotenone model (Green et al., 2009). In those same models, however, other authors found no significant differences in gastric transit, despite a reduction of dopaminergic TH-positive gastroenteric neurons (Anderson et al., 2007), suggesting that this result can be due to the higher expression of dopaminergic neurons in the upper GI tract compared to the distal portion, so that they can furnish a functional reserve (Li et al., 2006).

To better investigate these peripheral phenomena it has been chosen to damage the nigrostriatal tract administering the neurotoxin 6-OHDA centrally and selectively at the level of nigrostriatal pathway. In this model it is possible to assess whether the central lesion is responsible for the alterations that develop at the peripheral level, where the toxin can have no direct effect, unlike what occurs in the models with MPTP or rotenone, where the exposure to the neurotoxic agent occurs systemically. Although in these models the disease course is slower and shares some typical characteristics of the human disease, such as the development of Lewy bodies, the intraperitoneal administration of the toxin (MPTP or rotenone) does not allow to conclude whether the GI and urinary system alterations are due to local effects rather than to a central deficit and/or a deficit of communication between the CNS and PNS/ENS, making these models less suitable to the study.

The results obtained in our experimental model show that the 6-OHDA rats present disruptions of the gastrointestinal motility in different time points following the whole course of the experiments. The evaluation of radiopaque scores obtained from the x-ray imaging and measurement of total gastrointestinal transit as a contribution of the individual segments using Evan Blue as an unabsorbable dye allowed at different time points to identify which portions of the digestive tract develops dysmotility. The

application of the radiological methodology, adopted for the first time in this experimental model, has enabled us to highlight a temporal evolution of the GI motor disorders which were accentuated over the weeks from the time of the lesion (data already published in Vegezzi et al, 2014). The two mentioned methods showed no changes in GI motility after 4 weeks from central dopaminergic lesion, but a delay in the phase of emptying of the stomach and a reduced progression of the intraluminal contents in the small intestine after 8 weeks from surgery, as evidenced by the lower value of the geometric centre and the radiopaque scores. Furthermore, the radiological analysis evidences at 6 weeks an accelerated gastric emptying and ready filling of the small intestine within 2 hours from radiopaque gavage in 6-OHDA rats compared to controls.

Regarding the lower GI motility, the x-ray imaging showed a delay in the filling and emptying of the caecum and colorectum after 8 weeks from lesion, data which is confirmed by the extension of the t₂ period required for the complete emptying of the intestine of 6-OHDA rats compared to controls, as obtained by the orofecal transit evaluation. This latter data together with the measurement of the fecal output revealed that 6-OHDA rats develop constipation. Similar data are reported in the literature by other authors that, in the same model, detect a smaller amount of feces expelled, starting from three (Blandini et al., 2009) or four (Zhu et al, 2012) weeks after injury. Several hypotheses have been advanced to explain this phenomenon. Some authors (Colucci et al., 2012; Blandini et al., 2009) believe that the decreased expression of enteric n-NOS causes a slowing in ileal and colonic motility, and is accompanied by a down regulation of D2 receptors in the colon. Zhu suggests that the lack of n-NOS and the increase of dopamine neurons are also the basis of the slowdown observed in gastric emptying (Zhu et al., 2012). The lack of inhibitory component in some GI regions such as distal ileum and proximal colon, also induces an increased expression of VIP (Colucci et al., 2012), which, however, fails to restore motility. Further interpretation of the alteration of VIP and n-NOS can be traced back to the fact that nitric oxide and VIP are involved in neuronal survival and intestinal plasticity in the the presence of neurodegenerative diseases (Delgado et al., 2003).

Other authors attribute the alteration of GI transit to lack of acetylcholine (Zheng et al., 2011), although there is controversial evidence (Zhu et al., 2012; Colucci et al., 2012). Interestingly, the most noticeable changes to the GI motility occur in those areas where the vagal control is more important (Colucci et al., 2012).

Even dopamine seems to be one of the main factors responsible for constipation developed by patients with PD. The dopamine receptors are in fact distributed throughout the GI tract, and their number is decreased in the colon in patients (Singaram et al., 1995) as well as in the animal model of 6-OHDA (Colucci, 2012). The main role of dopamine is to relax the enteric smooth muscles, thus generating a retarding effect on progression of the chyme throughout the digestive tract (Schuurkes et al., 1981). The delay of transit observed in most of the patients could be caused by an increase in the presence of dopamine that through activation of the presynaptic receptor D2 leads to the suppression of acetylcholine release from nerve terminals (Hardoff et al., 2001). Zhu, through immunohistochemical studies, supports the hypothesis of an increased TH positive dopaminergic neurons and a parallel damage to n-NOS inhibitory pathways in the colon but also in the stomach, especially at the level of the antrum (Zhu et al, 2012).

Similarly Tian (2008), in the same experimental model, demonstrated an increased expression of the transporter for dopamine (and TH enzyme involved in its synthesis) at the level of the gastroenteric epithelial and neuronal cells. The expression of dopamine and its carrier are in fact increased in 6-OHDA model after four weeks, and the local increase of dopaminergic tone (Zhu et al., 2012) could explain the down regulation of D2 receptors reported by other authors in the colon (Colucci et al., 2012) and found, in functional terms in our research, at the level of the pylorus and in longitudinal strip from whole stomach. Indeed, we have observed that after 8 weeks from surgery the *in vitro* preparations of these two districts, isolated from animals with central dopaminergic lesion, respond to the relaxing stimulus by exogenous dopamine in a D2-independent way, in contrast with the animal control. Beside the stomach and pylorus, it has been detected a similar behaviour studying the colonic motility through the spatiotemporal organization of its motor patterns LDC (long distance contractions) and RPMCs (rhythmic propulsive motor complexes). While the colon of control animals responds to the D2R antagonist normally with an increase in propulsive contractions, showing an intact intrinsic dopaminergic activity, the colon of animals with central dopaminergic lesion induced over 8 weeks exhibits a negligible response. This suggests the occurrence of lack of expression of D2 receptors in the colon or their inactivation as consequence of central dopaminergic deficit.

Comparing the responses to electrical field stimulation of the circular gastric fundus of control and 6-OHDA animals, we observed in this latter a reduced neurogenic relaxation and a possible loss of an excitatory peptidergic component. These features can justify the gastric stasis and, in general, a reduced motility of the circular gastric fundus.

The examination of the responses of the pylorus in the two different groups of animals showed that the 6-OHDA tissues respond with a less prominent relaxation to electrical field stimulation that being NO independent reveals a loss of nitrenergic inhibitory innervation of this district. This data would complement the situation depicted by Colucci (2012), which has described a significant decrease in the expression of n-NOS in the lower GI tract of the 6-OHDA rats. The relaxation of the pylorus, in order to allow the passage of the chyme in the duodenum, is controlled by the action of nitrenergic enteric neurons (Sivarao et al., 2008) and the contribution of extrinsic projections of the vagus nerve (Greene et al., 2009). It is plausible to expect that the dysfunction of one of these regulation pathways leads to the delayed gastric emptying. The comparison of the responses to electrical field stimulation of gastric longitudinal strips of control and 6-OHDA rats highlighted, in the latter, the basal weak reaction to the stimulus in all the experimental conditions compared to controls.

A recent study conducted on rats lesioned with 6-OHDA in the right cerebral hemisphere (in analogy with our study), revealed at the level of the striatum an up-regulation of the postsynaptic D2 receptor and down-regulation of dopamine transporter (DAT) (Choi et al., 2012) interpreting the overexpression of the receptor as a consequence of the reduced presence of dopamine induced by the lesion itself. The main control centers of GI activity (representing the enteric extrinsic innervation) consist of the dorsal motor nucleus of the vagus (DMV) (parasympathetic component) and the nucleus of the solitary tract (NTS) (sympathetic component), both interconnected with the nigrostriatal pathway. Following the lesion with 6-OHDA, it has been measured a reduced presence of ChAT and increased expression of TH-positive neurons in the DMV of 6-OHDA rats, in addition to a reduction of the content of ACh in the gastric musculature. The neurons of the NTS exert an inhibitory action against the DMV preganglionic cholinergic neurons via release of catecholamines (Zheng et al., 2011). These alterations at the central and peripheral

level could explain the slowing of gastric emptying observed especially in the lesioned rats.

Part 2: Effects of oral subchronic treatment with L-DOPA / Benserazide

In this second part of the research it has been evaluated the evolution of gastrointestinal disorders following an oral subchronic four weeks treatment with L-DOPA/Benserazide (6 mg/kg e 15 mg/kg) after four weeks from the dopaminergic central lesion.

Despite the passage of more than 40 years since its first use, L-DOPA is considered a "gold standard" in the treatment of Parkinson's disease, in combination with Benserazide, a selective inhibitor of peripheral DOPA decarboxylase, which facilitates its access to CNS and reduces the dose to be administered and the eventual peripheral side effects.

When GI motility parameters have been studied in this experimental model after several weeks of drug treatment neither colonic motility nor orofecal transit have been modified significantly. But it seems to partially improve the condition of constipation as it can be observed from the daily fecal output but in a discontinuous way, more likely because of the few number of animals.

From a comparison of radiographic images related to the 6-OHDA rats and those receiving the subchronic treatment with L-DOPA/benserazide, it is possible to observe how such treatment prevented the delayed gastric emptying and partially reversed the delay in the filling of the caecum and colorectum displayed by 6-OHDA group compared to the control after 8th week from surgery.

Several recent reports suggest that in the pathogenesis of sporadic and familiar forms of PD, neuroinflammatory processes can interfere, besides oxidative stress, accumulation of abnormal proteins and protein aggregates, defects in the ubiquitin-proteasome system and mitochondrial damage particularly at the level of dopaminergic neurons (Kones R 2010). The increased vulnerability of dopaminergic neurons is explained by the formation of reactive species from dopamine metabolism, mitochondrial dysfunction and the resulting neuroinflammation (Hwang et al., 2013). The presence of a neuroinflammatory state with microglial activation is described in

the striatum and substantia nigra of patients with PD (Haddadi R. et al., 2013) whose brain shows an overexpression of myeloperoxidase, an enzyme involved in the formation of oxidants species by active microglia. Elevated levels of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6 were measured in glial cells, serum and in the brain and cerebrospinal fluid of patients with PD (Haddadi R. et al., 2013). These considerations have led us to assess whether conditions of oxidative stress could also occur at the enteric level in the experimental model of PD in coincidence with the increased activation of the dopaminergic system. The measurement of tissue levels of MDA, indicator of lipid peroxidation, revealed signs of oxidative stress more pronounced in regions of the GI tract characterized by major motor disorders such as stomach fundus and corpus, ileum and partially distal colon. It is noteworthy that the subchronic treatment with L-DOPA/Benserazide smoothed the development of dysmotility as well as the increase of MDA levels in the stomach and colon of 6-OHDA rats. This finding is a further evidence supporting a possible association between oxidative stress and impairment of regular GI motor processes, so that it becomes plausible to hypothesize for PD patients the usefulness of therapeutic interventions with protective antioxidants.

In this experimental model, it has been always possible to appreciate a reduction of water consumption by 6-OHDA rats compared to control for the whole course of the study. This interesting data may indicate the presence of adipsia, loss of thirst or abnormal abstinence from the intake of fluids, a symptom affecting individuals with PD (Ueki et al., 2004). The adipsia develops in a very remarkable way when the 6-OHDA lesion is bilateral (Sakai et al, 1994), so that the rats would not survive if not fed intragastrically. In our model of unilateral lesion the half integral part of the brain compensates almost completely to this large deficit, but leaving a constant behaviour to drink significantly less water. The reduction in the consumption of water is not going to affect the data on gastrointestinal transit. In fact the two phenomena occur at different times, supporting the hypothesis that the intestinal dysmotility and constipation are not due to a mere dehydration. Similarly, the data obtained from water consumption can explain the marked oliguria, statistically significant throughout the course of the experiment.

Part 3B: Role and involvement of ICC (Interstitial Cells of Cajal) in gastrointestinal motor dysfunctions associated with a central dopaminergic damage in a rat model of Parkinson's disease

The immunohistochemical studies showed interestingly for the first time in this PD model, a significant increase of ICC density in the fundus and antrum of the stomach in the 6-OHDA rats 4 and 8 weeks (except the corpus) after central dopaminergic deficit. This is accompanied by a similar significant increase of n-NOS expression in the same regions after 4 weeks and in the fundus after 8 weeks. From many extensive studies on the ICCs we know that they have a particular plasticity, with an ongoing loss and replacement in few days or weeks (Farrugia G. 2008) and the oxidative stress with a consequent proinflammatory response can easily damage these cells. In 6-OHDA gastric tissues appears that mastcells are intimately in proximity to ICCs, leading us to speculate on their role in stimulating the cells to proliferate. One of the interleukins produced by mastcells is Il-9, which is a growth factor and a stimulus of regeneration of the ICCs as indicated by recent evidences (Ye J et al., 2006). In addition to this, as regards the parallel increase of n-NOS expression, it is important to highlight not only the structural but also the functional interconnection between ICCs and nitrergic neurons and the fact that neuronally derived nitric oxide (NO) is considered a proliferation factor of ICCs (Farrugia G. 2008).

The variable changes in ICC density in the stomach of 6-OHDA rats seem to follow a similar temporal trend relatively to the nigrostriatal lesion, demonstrating that the central dopaminergic deficit have disrupted the normal physiology of these cells in the stomach (and not in the colon).

And similarly it happens for the n-NOS expression. The n-NOS positive area percentage is higher, 4 and 8 weeks after the central lesion, in the gastric regions with enhanced c-kit positive areas and in a similar extent.

The ICC density in 6-OHDA rats doesn't change in the colon comparing to the control after 4 and 8 weeks. While the NOS positive area density is increased after 4 and 8 weeks from surgery in the distal colon suggesting a differential cross-talk between nitrergic innervation and ICC in these gut regions. The absence of mast cells in the colon could account for this different behaviour.

There are evidences in literature which correlate oxidative stress and the iperexpression of n-NOS. Particularly Catania M.V. (2001) found an increased

expression of n-NOS (with an antibody raised against the C-terminal region of rat n-NOS) in specific brain regions affected by cell death caused by DNA damage and in reactive astrocytes in correspondence of beta amyloid plaques in patients affected by Alzheimer's disease, suggesting that NO released by glial cells might contribute to neuronal degeneration in different pathological conditions.

In conclusion, this research has provided significant results and methodological knowledge. Comparing different types of experimental procedures, it has been possible to identify the methods that, in this specific experimental model, displayed higher reproducibility and limited variability, allowing us to monitor in vivo the gastrointestinal motor activity.

It has been shown that the experimentally induced nigrostriatal dopaminergic deficit is associated with a temporal progressive change of the motor profile of specific regions of the GI tract, which reproduces the forms of GI dysmotility described in patients with PD.

Indeed, the derangement of GI motility detected 8 weeks after 6-OHDA cerebral injection is associated with immunoistochemical (n-NOS expression, ICC density), functional (neurogenic NO-dependent, Dopamine-induced and D2 receptor mediated relaxation) and biochemical (MDA levels) changes that differently affect the distinct portions of the digestive tract.

The stomach is particularly interested. Its delayed emptying seems to ensue from:

- loss of nitrenergic relaxant control of pyloric sphincter.
- reduced sensitivity of circular fundic musculature to exogenous contracting agents coupled with an increased n-NOS expression and nitrenergic responsiveness.
- fade of dopamine relaxing response with an apparent reduction in D2 receptor mediation.

The increased fundic levels of MDA and the changes of ICC density led us to speculate that gastric oxidative stress and modified ICC functions develop as a consequence of central dopaminergic neurodegeneration.

Moreover it has been observed that the subchronic treatment with L-DOPA / Benserazide change these motor dysfunction but in a discontinuous way.

Regarding the knowledge acquired in Dr Huizinga's Lab in Canada, it allowed to discover potential abnormalities of interstitial cells of Cajal in the 6-OHDA model of PD, not only quantitatively but also defining their location and connection on specific enteric neurons involved in the control of gut motility like nitrergic neurons.

The innovative data obtained during these three years are essential for the creation of a framework for future studies on patients with PD and on the treatment of this disorder. Lastly the findings obtained in the 6-OHDA model confirm their translational value since they can open new avenue to better understand the genesis of this disease with useful outcomes for the pharmacological treatment and for the identification of early marker predictors of PD.

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Partecipations in Congresses:

Zainab Al Harraq, Gaia Vegezzi, Francesca Miduri, Giacomo Gnudi, Giovanna Levandis, Silvia Cerri, Fabio Blandini, Giuseppe Domenichini, Simona Bertoni, Vigilio Ballabeni, Elisabetta Barocelli. "In vivo and in vitro investigation of upper gastrointestinal dysmotility in a model of Parkinson's disease". E-poster.
NMDPD The 10th International Congress on Non-Motor Dysfunctions in Parkinson's Disease and Related Disorders, Nice, France, 4-7 December 2014.

Al Harraq Z., Vegezzi G., Sacconi F., Domenichini G., Ballabeni V., Bertoni S., Gnudi G., Miduri F., Cerri C., Levandis G., Blandini F., Barocelli E. "Gastrointestinal symptoms and local oxidative stress in a model of Parkinson's Disease in rats".
Poster presentation.
36° SIF National Congress, Turin 23 -26 October 2013.

Vegezzi G., **Al Harraq Z.**, Sacconi F., Domenichini G., Ballabeni V., Gnudi G., Miduri F., Barocelli E. "Radiological analysis of gastrointestinal dysmotility associated with central dopaminergic impairment in an animal model of Parkinson's disease (PD)"
Poster presentation.
The 11th International Conference on Alzheimer's and Parkinson's Diseases (AD/PD)
Florence 06-10 March 2013.

Z. Al Harraq, F. Sacconi, G. Vegezzi, G. Domenichini, S. Bertoni, V. Ballabeni, E. Barocelli. "Characterization of gastrointestinal (GI) motor alterations associated with 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease in rats". Poster.
ULLA Summer School
School of Pharmacy, London, UK.
06-13 July 2013.

Z. Al Harraq, F. Sacconi, G. Vegezzi, G. Domenichini, S. Bertoni, V. Ballabeni, E. Barocelli. "Peripheral dysfunctions in a rat model of Parkinson's disease".
Oral presentation and poster.
16° National Seminars for PhD students and researchers, Rimini 16-19 September 2012.

Publications:

Vegezzi G¹, **Al Harraq Z¹**, Levandis G., Cerri S., Blandini F., Gnudi G., Miduri F., Blandizzi C., Domenichini G., Bertoni S., Ballabeni V., Barocelli E. "Radiological analysis of gastrointestinal dysmotility associated to a model of Parkinson's disease: comparative study with conventional in vivo techniques in the rat". J Pharmacol Toxicol Methods 2014 Sep-Oct; 70(2):163-9. ¹These authors contributed equally to this work and considered first authors.

Abroad Research Activity:

Research Fellowship in Neurogastroenterology and Motility

From 28-03-2014 to 3-11-2014

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Italian Society of Pharmacology (SIF) award (13/02/2014).