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Evaluation of the antioxidant action of the G protein-coupled estrogen receptor: contribution to the neuroprotective actions of the receptor

Ana Carolina de Matos Bernardino

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Orientadora: Prof. Doutora Graça Maria Fernandes Baltazar

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Dedicatória

Para a minha maior estrela no céu, o meu avô, que sempre me incentivou, apoiou e persuadiu a lutar pelos meus sonhos e por aquilo que realmente acredito.

"Someday, everything will make perfect sense.
So, for now, Laugh at the confusion,
Smile through the tears, and keep
reminding yourself that
everything happens for a reason.
Today will never come again.
Be a friend. Encourage
Someone. Take time to care.
Let your words heal and not
wound. Love, love, love,
Because Love conquers all! "

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Resumo

O cérebro caracteriza-se por apresentar um elevado metabolismo, e contém várias substâncias facilmente oxidáveis, tais como aminas e lípidos, o que resulta numa exposição a elevados níveis de stress oxidativo. Foi demonstrado que na doença de Parkinson (DP), o stress oxidativo está correlacionado com a peroxidação lipídica, inflamação, disfunção mitocondrial e agregação da α-synucleína (α-syn). Isto demonstra que o stress oxidativo pode ser um dos desencadeadores da doença de Parkinson, por ser capaz de induzir uma série de mecanismos patogénicos característicos da doença, contribuindo de forma crucial para a sua progressão. Neste sentido, a identificação de mecanismos que ajudem a reduzir o stress oxidativo podem ser estratégias interessantes para o controlo da progressão da doença. Uma vez que o 17β-estradiol foi classificado como neuroprotetor e já demonstrou efeitos benéficos em diversos mecanismos como neuroinflamação, excitotoxicidade, entre outros, fomos avaliar se a ativação seletiva do recetor de estrogénios acoplado à proteína G (GPER), caracterizado por estar envolvido em ações não genómicas rápidas do 17β-estradiol, pode exercer um efeito neuroprotetor associado à modulação do stress oxidativo na DP. Com este objetivo, desenvolvemos um estudo *in vivo* com murganhos injetados com 6-OHDA, que foram, posteriormente, submetidos a tratamento subcutâneo ou intranasal com um agonista do recetor, o G1. Assim, avaliámos de que forma a ativação seletiva do recetor pode contribuir para a reversão do stress oxidativo. Para isso, foram efetuados vários testes comportamentais para avaliar a função motora, como o Grip Test, o Rotarod e o Open Field Test, e foram medidos os níveis de mRNA de enzimas antioxidantes, por PCR em tempo real (RT-PCR). A partir dos testes comportamentais, foi possível concluir que a injeção da toxina não afetou o comportamento motor uma vez que os resultados obtidos no Rotarod, e distância total percorrida obtida no Open Field Test não mostraram diferenças significativas. Por outro lado, foi possível observar que a injeção com 6-OHDA aumentou os parâmetros relacionados com o comportamento ansioso. Desta forma, é possível concluir que a toxina não exerceu efeito ao nível do comportamento motor, porém, induziu alterações a nível não-motor. Relativamente à expressão das enzimas antioxidantes, observou-se um aumento, ainda que sem significância estatística, dos níveis de mRNA da Gpx4 e do Nrf2 em animais injetados com 6-OHDA. Este aumento, pode querer evidenciar um mecanismo de proteção desencadeado por estas enzimas para lidar com o stress oxidativo. No entanto, mais estudos seriam necessários para conseguir comprovar esta hipótese. Os nossos resultados evidenciaram efeitos exercidos pelo G1, quando entregue pelos dois tipos de administração. No entanto, não foi possível concluir se os dois tipos de

entrega do G1 têm um efeito antioxidante na presença de um insulto dopaminérgico. Neste sentido, mais estudos seriam necessários para perceber se a ativação do GPER é capaz de modular o stress oxidativo e, se este efeito está relacionado com os seus efeitos neuroprotetores atualmente reconhecidos.

Palavras-chave

17β-estradiol; Doença de Parkinson; Enzimas antioxidantes; Recetor de Estrogénios acoplado à proteína G; Stress Oxidativo.

Resumo Alargado

A doença de Parkinson (DP) é atualmente considerada a segunda doença neurodegenerativa mais prevalente em todo o mundo. O cérebro apresenta um metabolismo elevado, e contém várias substâncias facilmente oxidáveis, tais como as aminas e lípidos, o que resulta numa exposição a elevados níveis de stress oxidativo. Na DP foi demonstrado que o stress oxidativo está correlacionado com a peroxidação lipídica, inflamação, disfunção mitocondrial e agregação da α-synucleína (α-syn). Enquanto que em condições fisiológicas, a SNc apresenta um declínio dependente da idade nos mecanismos antioxidantes, na DP ocorre um declínio geral de todo o sistema antioxidante, resultando num desequilíbrio oxidativo. Este desequilíbrio, é caracterizado pelo aumento de espécies reativas e pela diminuição de defesas antioxidantes, originando a acumulação de espécies reativas de oxigénio. Isto demonstra que o stress oxidativo pode ser um dos principais mecanismos desencadeadores da doença por ser capaz de induzir uma série de mecanismos patogénicos característicos da doença, contribuindo de forma crucial para a sua progressão. Uma vez que dados demonstram que o 17β-estradiol exerce uma função neuroprotetora e já foram demonstrados os seus efeitos benéficos em mecanismos como a neuroinflamação, excitotoxicidade, entre outros, fomos avaliar se a ativação seletiva do recetor de estrogénios acoplado à proteína G (GPER), caracterizado por estar envolvido em ações não genómicas rápidas do 17β-estradiol e por se interrelacionar com outros tipos de recetores de estrogénios como o ER- α-36 e o Erα, pode exercer um efeito neuroprotetor associado à modulação do stress oxidativo na DP.

Com este objetivo, desenvolvemos um estudo *in vivo* com murganhos aos quais foi injetada 6-OHDA. Esta toxina induz aumento do stress oxidativo por inibição do complexo I da cadeia de transporte de eletrões. Os animais foram, posteriormente, submetidos a administração subcutânea ou intranasal de um agonista do recetor GPER, o G1 como o objetivo de avaliar de que forma a ativação seletiva do GPER pode contribuir para a modulação do stress oxidativo característico da DP. Recorremos a diversos testes comportamentais com o objetivo de avaliar a função motora, como o Grip Test, o Rotarod e o Open Field Test. A partir destes, foi possível concluir que a injeção da toxina não afetou o comportamento motor uma vez que os resultados obtidos no Rotarod, assim como a distância total percorrida, medida no Open field Test, não apresentaram diferenças significativas. Por outro lado, foi possível observar que a injeção com 6-OHDA aumentou os parâmetros relacionados com o comportamento ansioso. Desta forma, é possível concluir que a toxina não exerceu efeito ao nível do comportamento motor dos animais, porém, induziu alterações a nível não-motor. Foram ainda medidos os níveis relativos de mRNA

das enzimas antioxidantes SOD1, CAT, Gpx4 e do fator de transcrição Nrf2 por PCR em tempo real (RT-PCR). Relativamente à expressão destes, observou-se um aumento, ainda que sem significância estatística, dos níveis de mRNA do Nrf2 e da Gpx4 em animais injetados com 6-OHDA. Este aumento, pode querer sugerir um mecanismo de proteção no sentido de promover a transcrição de enzimas antioxidantes e de preservar a viabilidade neuronal, tentando limitar os níveis de stress oxidativo. Os resultados obtidos evidenciam efeitos exercidos pelo agonista do recetor pelos dois tipos de administração, no entanto, através destes, não é possível concluir se as duas abordagens de entrega do G1 exercem um efeito antioxidante na presença de um insulto dopaminérgico. Deste modo, seria necessário desenvolver mais estudos para perceber se a ativação do GPER é capaz de modular o stress oxidativo e, se este efeito está relacionado com os seus efeitos neuroprotetores atualmente reconhecidos.

Palavras-chave

17β-estradiol; Doença de Parkinson; Enzimas antioxidantes; Recetor de Estrogénios acoplado à proteína G; Stress Oxidativo.

Abstract

The brain is characterized by a high metabolism and contains several easily oxidizable substances such as amines and lipids, resulting in exposure to high levels of oxidative stress. In Parkinson's disease (PD), oxidative stress has been shown to be correlated with lipid peroxidation, inflammation, mitochondrial dysfunction and aggregation of α-synuclein (α-syn). This demonstrates that oxidative stress can be one of the triggers of Parkinson's disease, as it is capable of inducing a series of pathogenic mechanisms characteristic of the disease, contributing to its progression. In this sense, the identification of mechanisms that help reducing oxidative stress may be an interesting strategy for controlling the progression of the disease. Since 17β-estradiol exerts neuroprotective functions and has proved beneficial effects on several mechanisms such as neuroinflammation, excitotoxicity, among others, we assessed whether the selective activation of the G protein-coupled estrogen receptor (GPER), characterized by being involved in rapid non-genomic actions of 17β-estradiol, can exert a neuroprotective effect associated with the modulation of oxidative stress. With this objective, we developed an *in vivo* study with mice injected with 6-OHDA, which were later submitted to subcutaneous or intranasal treatment with the GPER agonist, G1. We evaluated how the selective activation of the receptor can contribute to the reversion of oxidative stress. To this end, several behavioral tests were performed to evaluate motor function, such as Grip Test, Rotarod and Open Field Test, and relative mRNA levels of antioxidant enzymes were measured by real-time PCR (RT-PCR). From the behavioral tests, it was possible to conclude that the 6-OHDA-injection was not capable of affecting motor behavior, since the results obtained with the Rotarod test, and the total distance travelled obtained with the Open field Test did not present significant differences. On the other hand, it was possible to observe that the parameters related with anxious behavior were increased in animals injected with 6-OHDA, when compared with the control group. Therefore, it can be concluded that the toxin had no effect at the level of motor behavior, but induced changes in non-motor domains. Regarding the expression of antioxidant enzymes, although not significant, an increase in the mRNA levels of Gpx4 and Nrf2 was observed in 6-OHDA-injected mice. This increase suggests a protective mechanism aiming to limit oxidative stress. However, further studies are needed to confirm this hypothesis. Our results have shown effects exercised by the G1, when administered by the two delivery approaches. However, it was not possible to conclude whether the two types of G1 delivery have an antioxidant effect in the presence of a dopaminergic insult. In this sense, further studies would be necessary to confirm whether GPER activation is capable of modulating oxidative stress and whether this effect is related to its currently recognized neuroprotective effects.

Keywords

Antioxidant enzymes; Estrogens; G protein-coupled estrogen receptor; Oxidative Stress; Parkinson's Disease.

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List of Abbreviations

PD	Parkinson's disease
DA	Dopaminergic
SNc	*Substantia nigra pars compacta*
STR	*Striatum*
α-syn	α-synuclein
LRRK2	Leucine rich repeat kinase 2
PINK1	PTEN-induced putative kinase 1
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
MPTP	1-methyl-4-phenyl-1,2,3,4-tertahydropyridine
MAO	Monoamine Oxidase
iPSC	Pluripotent stem cells
SOD	Superoxide dismutase
CAT	Catalase
GSH	Glutathione
GPx	Glutathione peroxidase
NOX	NADPH oxidase
Nrf2	Transcription factor NF-E2 related protein factor 2
Keap1	Kelch-like ECH-associated protein 1
E2	17β-Estradiol
GPER	G Protein-Coupled Estrogen Receptor
EREs	Estrogen response elements
GPCR	G Protein-coupled receptor
TH	tyrosine hydroxylase
6-OHDA	6-Hydroxydopamine

Chapter 1

Introduction

Introduction

1.1. Etiology and pathology of Parkinson's Disease

Parkinson's disease (PD) was first described in 1817 by James Parkinson in his monograph "An Essay of the Shaking Palsy" and is presently considered the second most prevalent neurodegenerative disorder worldwide.[1] Pathologically, PD is characterized by the loss of dopaminergic (DA) neurons in *Substantia Nigra pars Compacta* (SNc) that project to the *Striatum* (STR), and by the accumulation of cytoplasmatic protein inclusions known as Lewy bodies or Lewy neurites.[2] Dopamine is a catecholamine neurotransmitter involved in cognitive functions, motor control, memory, emotions, neuroendocrine regulation and reward mechanism.[3] The existence of dopaminergic receptors was demonstrated in 1972, when dopamine was shown to stimulate the activity of adenylcyclase.[4,5] Later, the receptors were classified as type D1 (D1 and D5) and D2 (D2, D3 and D4).[6] The most affected area of the SNc in PD is the ventrolateral tier, composed by neurons that project to the dorsal putamen of the STR, which may justify some of the symptoms present in PD, such as bradykinesia. The characteristic loss of neurons in PD occurs in many other regions including amygdala, hypothalamus, locus coeruleus, nucleus basalis of Meynert, dorsal motor nucleus of the vagus and pedunculopontine and raphe nucleus.[7]

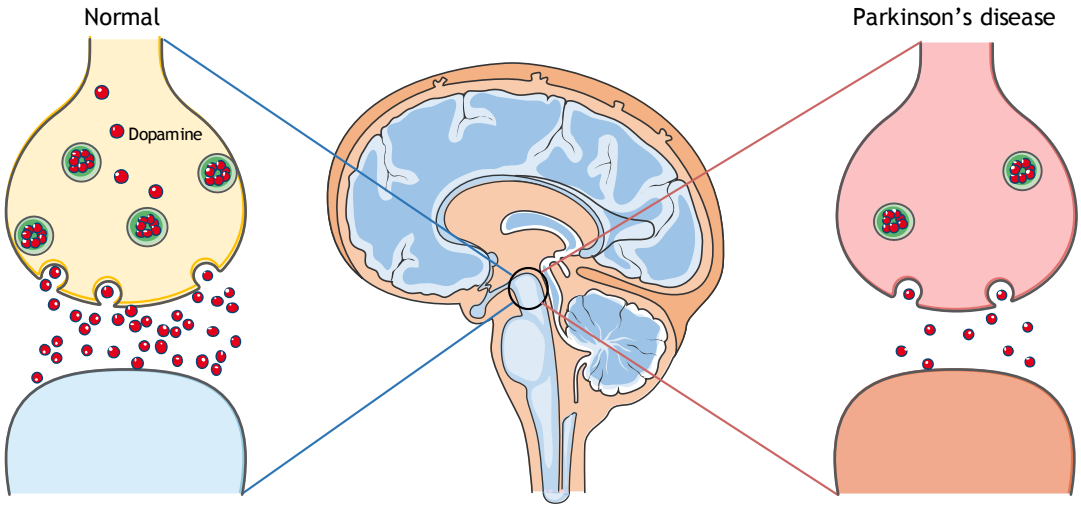

Figure 1. Schematic representation of dopamine loss in Parkinson's disease.

The decrease in dopamine levels in the striatum result mainly in motor symptoms such as tremor, slow body movement and muscle rigidity.[8,9] It is believed that 50-70% of SNc dopaminergic neurons had already died when motor clinical symptoms became evident.[10] However, non-motor symptoms such as sensory deficiency, gastrointestinal and bladder dysfunction, psychiatric alterations, fatigue, and sleep disturbances are also present in this

neurodegenerative disease.[11,12,13,14,15] In later stages of the disease, additional non-motor symptoms such as autonomic dysfunction, pain and cognitive decline may occur.[9] In addition, studies have shown that PD development can severely affect other neurotransmitter systems such as the serotonergic, noradrenergic, and cholinergic systems.[16,17,18] In fact, post-mortem studies have also shown that serotonergic neurons are affected by PD, showing a significant reduction in serotonin carrier density (SERT), especially in caudate and putamen. [16,18,19]

Although its cause is not known, Parkinson's disease seems to have a direct link with age, gender, genetic and environmental factors. Previous studies have shown that there are clear differences in the epidemiological and clinical characteristics of the disease, related to the gender of the patient. PD affects men more often than women, which has been related to the neuroprotective effect of estrogens.[20,21,22] Besides, studies have shown that decreased estrogen levels in older women, early menopause or ovarian removal increases the risk of Parkinson's development.[23,24]

Aging is the biggest risk factor for the development of PD. Evidences confirm that sporadic PD is rare prior to 50 years, however, prevalence steadily increases to 2% in the global population aged 65 years, peaking 5% in individuals aged 80 years. This association suggests that age-related biomolecular changes in regions of the brain that are vulnerable to degeneration in PD, specifically in SNc, contribute to an increased risk of PD development.[25]

In addition, there are other factors that seem to contribute to the development of Parkinson's disease, for instance, genetic and environmental factors such as pesticide exposure, prior head injury, β-blocker use, among others.[26] The genetic contribution to the development of this disease seems to be associated with a family history of PD and tremor.[26] The first gene associated with inherited Parkinson's disease was the *SNCA*, which encodes the protein α-synuclein (α-syn).[27] Other well-known mutations is in leucine rich repeat kinase 2 (LRRK2) and parkin, which cause the dominant and recessive inheritance of PD, respectively.[28] Mutations in parkin and PTEN-induced putative kinase 1 (PINK1) genes are associated with early onset autosomal-recessive PD.[29,30] Both of these genes are related to pathways that involves the preferential degradation of mitochondria by dysfunctional lysosomes through macroautophagy, a process called mitophagy. Thus, the loss of function of these genes leads to deficient mitophagy, resulting in the accumulation of dysfunctional mitochondria. Indirectly parkin is also responsible for regulating PGC-1α levels. This transcriptional regulator modulates, in a coordinated manner, the expression of genes necessary for mitochondrial biogenesis, as well as multiple antioxidant defenses.[31]

However, glucocerebrosidase (GBA) gene mutations are currently considered the strongest genetic risk factor for PD.[32],[33] Although mutations in the GBA gene are usually associated with autosomal recessive Gaucher's disease, carriers of a GBA mutation have also a 4 times higher risk of developing PD.[34]

Currently there is no cure for this disease. There are only therapies that provide symptomatic relief and do not halt the neurodegenerative process. The best known and most used therapy for PD is the Levodopa (L-DOPA) treatment, but it loses effectiveness in advanced stages of the disease, and its chronic use can result in the development of motor complications, often termed as L-DOPA–induced dyskinesia, dystonia and hallucinations.[35,36] Dopamine receptor agonists, although less effective than L-DOPA, are also used in the early stage of the disease with the aim of controlling the symptoms and delaying the use, and the adverse effects, of L-Dopa.[37]

1.2. Neurodegenerative mechanisms involved in PD

1.2.1. Dysfunction in protein degradation pathways and α-Synuclein accumulation

α-syn is a small protein highly expressed in peripheral tissues and blood.[38] Under normal conditions, modulates the stability of the neuronal membrane and also influences the presynaptic signaling and membrane trafficking through vesicular transport.[39] This may be important given the association of α-syn with nerve terminal SNARE complexes, and suggests a potential role for this protein in modulating dopamine release.[40] Post-translational modifications, mutations or changes in the surrounding environment may lead to disruption of native protein conformation and induce misfolding and aggregation.[41,42] Diseases neuropathologically characterized by the accumulation of α-syn, can be designated as α-synucleinopathies.

1.2.2. α-Synuclein in Lewy neurites and Lewy bodies

Lewy bodies and Lewy neurites are small abnormal inclusions that accumulate in neurons in neurodegenerative diseases, such as PD. These cytoplasmatic inclusions are the main pathogenic marks in brain biopsies of PD patients and are not present in healthy individuals. Under pathological conditions, α-syn forms aggregates through the assembly of soluble oligomeric intermediates that mature in the insoluble amyloid fibrils found in Lewy bodies. Thereby, the major component of these bodies are insoluble α-syn fibrils[43], with the majority occuring within the pigmented neurons in the SNc.[7] Lewy inclusions are found in the remaining dopamine neurons and are described as round, intraneuronal, eosinophilic inclusions with a hyaline nucleus and a pale peripheral halo and are always positive for α-syn and ubiquitin.[44] The presence of Lewy's neurites seems to affect axonal transport and other fundamental cellular processes, thus compromising normal function and neuronal survival.[45] It is now widely recognized that α-syn aggregates can contribute to the progression and development of the disease by spreading throughout the central nervous system possibly in a prion-like manner.[46]

1.2.3. Excitotoxicity

Excitotoxicity is characterized by excessive stimulation of glutamatergic receptors, leading to death or injury of neurons. This process is involved in several brain diseases such as stroke and neurodegenerative diseases such as PD.[47] The degeneration of DA neurons in SNc induces an increase in the activity of glutamatergic neurons of the subthalamic nucleus (STN), which innervates the SNc and the internal segment of *globus pallidus*. As the disease progresses, and the dopamine levels decrease, neuronal STN activity becomes abnormal[48], possibly even before the appearance of clinical signs.[49] Glutamate is considered the most abundant neurotransmitter in the brain and its overstimulation increases intracellular Ca^{2+} levels, triggering a cascade of events leading to excitotoxicity.[50] The increase in cytoplasmic Ca^{2+} activates a number of Ca^{2+}-dependent enzymes involved in the catabolism of nucleic acids, phospholipids and proteins leading to necrotic cell death through different pathways, such as cytoskeleton changes in the nitric-oxide-derived free radical, membrane disruption, and also triggers apoptosis.[51] Although glutamate-mediated neurotoxicity is not responsible for the initial insult and neuronal loss in SNc, the intrinsic vulnerability of dopaminergic neurons, associated with scarce antioxidant defenses, mitochondrial deficiencies and aberrant proteolytic machinery, represents the perfect background for the activation of excitotoxic phenomena and, consequently, progression of nigrostriatal death.

1.2.4. Neuroinflammation

The inflammatory process is considered a protective mechanism in which there is repair and regeneration of damaged cells and tissues by removing infectious agents and toxins from the body. Some of the cells responsible for inflammatory responses are mast cells, macrophages, T cells, neutrophils and microglia.[52] In the central nervous system, inflammation is a protective mechanism that restores damaged glial and neuronal cells. However, when it becomes an excessive or repetitive process it can inhibit neuronal regeneration.[53] For a long time, the brain and the immune system were considered two isolated and independent entities, classifying the brain as an immuno-privileged organ.[54] However, currently, neuroinflammation is recognized to play a central role in the central nervous system in some diseases, such as PD. Pre-clinical and clinical studies have shown a relationship between neurodegenerative diseases, immune system activation and neuroinflammation.[55] In both animal and human models of PD, neuroinflammatory aspects are represented by activated microglia, reactive astrocytes, overexpression of pro-inflammatory cytokines, involvement of the innate and adaptive immune system and

increased concentrations of reactive oxygen and nitrogen species (ROS/RNS).[56,57] Although activation of microglial cells is beneficial for neuronal tissue by promoting the elimination of cell debris and causing the secretion of various neurotrophic factors, it can also be harmful since inflammatory mediators modulate immune cells and act on neurons, and can to contribute to neurodegenerative effects.[58]

Microglia functions are the first line of immunological response to pathological conditions in the Central Nervous System (CNS). After injury microglia changes from a surveillant state to a reactive state, altering their cellular morphology and phenotype that vary based on the type of the stimulus to which they are exposed[59] and respond by synthesizing and releasing inflammatory molecules (chemokines, interferons, interleukins, lymphokines and tumour necrosis factor), alert other immune cells and provide nutrients to repair the damage induced in the cells surrounding the inflammatory battlefield.[60] The neuronal cell death in the SNc of post-mortem PD patient has been found to be accompanied by significant microglial activation.[61] In this way, in living PD patients, activated microglia and significant death of DA neurons in the SNc were observed by the positron emission tomographic (PET) analysis.[62] Elevated expressions of pro-inflammatory TNF-α, β, cytokines IL-1b, IL-6 and pro-oxidant NOS, were also detected in the cerebrospinal fluid (CSF), *SNc*, putamen and serum of PD patients. [63]

1.2.5. Oxidative Stress

Since the brain has a high metabolism it is continuously exposed to high levels of oxidative stress.[64] Oxidative damage arises when there is a significant accumulation of ROS or RNS, either from overproduction of these species or from a reduction in the endogenous antioxidant capacity. The predominant site of ROS production is the mitochondria and its generation is initiated in mitochondrial complex I, through molecular oxygen (O_2) partial reduction to superoxide radical (O_2^{*-}) by a single electron uptake. In turn, it can lead to either hydroxyl radical (OH^*) generation through the Fenton reaction or to peroxynitrite ($ONOO^-$). Superoxide radical can also be converted to hydrogen peroxide (H_2O_2) and subsequently broken down to H_2O and O_2. At physiological levels, ROS contribute to fundamental cellular and molecular processes, such as cellular differentiation, cellular signaling, motility, growth and apoptosis. However, in excessive concentrations, ROS can cause lipid peroxidation, DNA damage, inflammation and protein modification that later lead to cellular dysfunctions and cell death.[65] It has been shown that in PD, oxidative stress is able to trigger lipid peroxidation, inflammation, mitochondrial dysfunction and induce

α-syn misfolding, modification and aggregation. Different studies show that oxidative stress exacerbates α-syn aggregation *in vivo* and induces the formation of α-syn aggregates in vitro.[66,67] It has also been shown that in brains of PD patients the production of malondialdehyde resulting from lipid peroxidation was highly increased in the SNc, when compared to healthy human brains.[68] Normally, endogenous aldehydes, such as malondialdehyde, are kept at physiological levels. Nevertheless, when the antioxidant function is reduced or dysregulated, they can accumulate and cause damage to the body, especially to the nervous system.[69,70] Oxidative stress induced by ROS and RNS can trigger reactivity of glial cells and inflammation and inhibit complex I activity.[71,72] This demonstrates that oxidative stress may be the main trigger for PD as it is able to induce a number of pathogenic mechanisms characteristic of the disease, contributing crucially to disease progression.[73]

1.2.6. Mitochondrial dysfunction and oxidative stress

Mitochondrial dysfunction is considered a crucial factor in PD and is mainly caused by anomalies in the mitochondrial electron transport chain complex I, homeostasis alterations and genetic mutations.[74] Histology of post-mortem brains of PD patients showed signs of mitochondrial dysfunction.[75] Furthermore the dopaminergic toxin 1-methyl-4-phenyl-1,2,3,4-tertahydropyridine (MPTP), frequently use to induce Parkinsonism in animal models, acts by inducing mitochondrial complex I inhibition.[76,77] Brain dopamine is oxidized either by self-oxidation or by monoamine oxidases (MAO) linked to the mitochondrial external membrane, such as MAO-A and MAO-B, resulting in dopamine quinones and free radicals.[78] Since oxidative stress increases the occurrence of mutations, it is possible that the excess of free radicals observed in PD contribute to mutations that make cells more susceptible to various dysfunctions. The presence of abnormal mitochondria was also demonstrated in pluripotent stem cells (iPSC)-derived from DA neurons from patients with PINK1 or Parkin mutations.[79] In another study using iPSC carrying a mutation in LRRK2 gene, a high expression of genes involved in the regulation of oxidative stress and on α-syn levels was observed.[80] PD-related genes such as PINK1, Parkin, DJ-1 and LRRK2 encode proteins that regulate mitochondrial homeostasis and ROS formation. PINK1 is a protein that, under normal conditions, is rapidly degraded by mitochondria. However, in the presence of increased oxidative stress, defective mitochondria, decreased membrane potential or protein misfolding, its degradation is inhibited, which leads to its accumulation. PINK1 is responsible for Parkin phosphorylation, a ubiquitin ligase that adds ubiquitin chains to proteins in the mitochondrial membrane

which act as signals for autophagy. These defects in mitophagy, and increased oxidative stress may be related to specific DA neurons phenotypes.[79,81,82] Another protein usually associated with PD is Dj-1. This protein has a cysteine residue highly susceptible to oxidation. The oxidation of this cysteine residue leads to cysteine-sulfinic acid formation, that contributes to oxidative stress.[83] LRRK2, also known as dardarin, is an enzyme responsible for mitochondrial morphology and for the regulation of autophagy. Mutations in LRRK2 are reported to induce synaptic dysfunctions in human dopaminergic neurons.[84]

1.2.7. Antioxidant dysfunction

The severity of symptoms observed in Parkinson's patients seems to be related to the imbalance between excessive production of ROS and down-regulated expression of endogenous antioxidant systems such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx), and NADPH oxidase (NOX).[85,86] All these enzymes are part of the endogenous system capable of reducing reactive oxygen species and consequently decrease toxicity. SOD is responsible for catalyzing the conversion of the highly toxic $O2^{-\cdot}$ into H_2O_2 and O_2, being H_2O_2 converted to H_2O and O_2 by CAT. GSH is crucial to the control of ROS since it has the ability to react with ROS generating glutathione disulfide (GSSG). A decrease in the levels of reduced glutathione, and a reduction in SOD, GPx, and glutathione reductase activities was observed in the post-mortem SNc of healthy aged individuals compared with younger individuals which indicates that this antioxidant mechanism my by compromised in healthy aging.[69]

NOX, a multi-subunit enzyme, is a major source of ROS and plays an important role in dopamine neurotoxicity. Although there are several NOX isoforms, their contribution to PD has not yet been fully disclosed. However, it has been demonstrated that the expression of NOX1 is reduced in SNc of PD patients.[87] Furthermore, there seems to be a crosstalk between neuronal NOX such as NOX1 and the microglial NOX2 responsible for intensifying neuronal injury and neuro-inflammatory response.[88]

On the other hand, Nrf2 is present in all human organs and is one of the main defenses against oxidative stress. As a redox-sensitive transcription factor, mainly modulates the activation of biological systems encompassing anti-inflammatory molecules, antioxidants (glutathione, among others), enzymes (such as cytochrome P450s), and free radical scavengers.[89,90] Nrf2 is characterized by being a short-lived protein that is normally subjected to continuous ubiquitination and proteasomal degradation. Its cytosolic

repressor, Kelch-like ECH-associated protein 1 (Keap1) senses oxidative stress in the cell, resulting in proteasomal degradation. The presence of oxidative stress causes the disjunction between Nrf2 and Keap1 and originates its translocation to the nucleus, where it will trigger a cellular response.[91] Thus, the Keap1-Nrf2 complex act as a redox sensor maintaining homeostasis and regulating the transcription of antioxidant genes.[92] Failure in the regulation of transcriptional activity by Nrf2 makes dopaminergic neurons susceptible to oxidative stress damage.[93] Activation of Nrf2 inhibits neurodegenerative events, whereas its inhibition accelerates this process.[94]

While healthy SNc shows an age-dependent decline in antioxidants mechanisms, PD is characterized by a general and abrupt decline in the entire antioxidant system resulting in a severe worsening of ROS production and disease progression.[95] Also, the presence of 4-hydroxynonenal from oxidative lipid degradation, by interacting with various proteins impairs cellular viability.[96] Lastly, oxidized adducts of nucleic acid (DNA and RNA) such as 8-Hydroxyguanosine (8-OHG) and 8-Hydroxy-2'-Deoxyguanosine (8-OHdG) are also increased in the SN and cerebrospinal fluid (CSF).[75]

1.3. The Role of Estrogens in PD

Sex impacts in the incidence, severity and progression of PD.[97] These differences are believed to be related to steroid hormones. A recent meta-analysis has shown difference between gender in the development of Parkinson's disease. Men aged 60-69 have significantly higher incidence rates (58.22 per 100,000 individuals/year) than women of the same age range (30.32 per 100,000 individuals/year). Similarly, men aged 70-79 also had significantly higher incidence rates (162.58 per 100,000 individuals/year) compared to women (93.32 per 100,000 individuals/year).[22] The higher incidence rate of the disease in men compared to women is probably related to higher estrogenic activity in women. Estrogens are known to protect the nigrostriatal dopaminergic pathway.[98,99] Estrogens are a group of hormones which includes estrone, estradiol, estriol and which, due to their central structure with carbon-carbon bonds arranged as fused rings, are classified as "steroids".[100] Estradiol (E2) has two isomers, 17α - estradiol and 17β- estradiol, being the former recognized as having higher estrogenic effect since it has very high affinity for estrogen receptors.[101] Steroid synthesis occur in gonads, adrenal cortex and placenta. However, in 1849 the physiologist Arnold Adolf Berthold discovered that this synthesis could also occur in the brain.[102] This synthesis starts from molecules such as cholesterol or steroid precursors. In mitochondria, cerebral cholesterol is converted into pregnenolone by the cytochrome cleavage enzyme (P450scc) and later metabolized in the endoplasmic reticulum, where it gives rise to neurosteroids such as progesterone, allopregnanolone (Allop), dehydroepiandrosterone (DHEA) or estradiol (E_2).[103]

1.3.1. Estrogens and its receptors

The first estrogen receptor was discovered by Elwood Jensen in 1958.[104,105] Known as Erα, is manly expressed in breast gland, uterus, ovary, bones, male reproductive organs, liver and adipose tissue. Whereas ERβ is mainly found in ovary, bladder, colon, adipose tissue and immunologic system. In contrast with ERβ, which has more profound effect on the central nervous and immune systems, Erα seems to play a critical role in the mammary gland and uterus. Besides that, it is critical in skeletal homeostasis and the regulation of metabolism.[106,107] A new estrogen-binding protein, estrogen receptor-coupled to protein G (GPER or GPR30) was identified in the late 1990s by Filardo and collaborators.[108] The role of GPER in mediating fast responses to estrogens has been studied in *in vivo* and *in vitro* models.[109,110] More recently, Wang et al identified a new subtype of estrogen receptor named ER-α-36. This receptor, derived from the classic ERα, is preferably localized on the plasma

membrane and cytoplasm.[111,112] GPER acts as a co-regulator of the ER-α-36 by physical interaction in the cytoplasm and possibly in the perinuclear area.[112] Estrogens, as steroid compounds, can interact directly with Erα and ERβ or can activate intracellular signaling cascades through interaction with GPER or Erα/ERβ. Since estrogen-receptor complexes can bind directly or indirectly to DNA, estrogen-mediated effects can be genomic or non-genomic. The genomic effects are those where Erα and Erβ act as ligand-activated transcriptional factors.[113] The genomic mechanism involve the migration of estrogen-receptor complexes to the nucleus, where they interact with chromatin in specific sites known as estrogens response elements (EREs).[114] The non-genomic effects may involve indirect regulation of gene expression through various intracellular signaling pathways.[115,116,117,118]

Through receptors highly expressed in the cardiovascular system[119,120], brain[60,121], adipose tissue[122], liver[123], prostate[124], testicles[125,126], among others, estrogens regulate multiple physiologic functions in both men and women.

1.3.2. GPER

GPER is a member of the G protein-coupled receptor (GPCR) family and was first discovered in 1996 in breast cancer tissue[127]. Its cDNA sequence was cloned in 1997 through differential cDNA library analysis of the human breast adenocarcinoma cell lines MDA-MB-231 and MCF7.[128] This receptor is expressed in many human and rodent tissues such as brain, placenta, lung, liver, ovary, pancreatic islets as well as immune cells.[129] The expression pattern of GPER is species-, age-, tissue-, or gender-dependent. For example, the mRNA expression of GPER in skeletal muscle tends to be higher in pre-menopausal women compared to post-menopausal women.[130] As a seven transmembrane GPCR, it is located on the plasma membrane[131–133], with a larger fraction of total cellular GPER being localized in intracellular membranes of the endoplasmatic reticulum[134,135] and Golgi apparatus[135]. The use of E2 fluorescent derivatives to visualize the extra and intracellular binding properties of GPER in monkey kidney fibroblasts (COS-7) demonstrated that the E2 derivatives were not able to bind to the plasma membrane and predominantly bound to endoplasmic reticulum.[110] GPER is also present in mitochondria in undifferentiated myoblasts of C2C12, and in the cytoplasm in differentiated myotubes of C2C12.[136] In neurons, GPER is located at extranuclear sites, including the plasma membrane, endoplasmatic reticulum and Golgi complex.[137,138] GPER is characterized by its involvement in rapid nongenomic actions and also in transcriptional effects of estradiol.[139] Although

different from other receptors in many respects, GPER is similar in protein G coupling, binding subunits Gα and Gβγ to activate intracellular signaling cascades, including cAMP generation and ERK1/2 transactivation[140] and synthesis of phosphatidylinositol 3,4,5-triphosphate in the nucleus[110,141] (figure 2). The most widely used GPER-selective ligands are the tetrahydroquinolines G1 (agonist)[142], G15[143] and G36[144] (both antagonists). G1 triggers many of the E2 effects, e.g. those associated with rapid signaling in models of traumatic brain and spinal cord injury[145], atherosclerosis[146], among others. On the other hand, G15 and G36 have been shown to have important applications in carcinogenesis[147] and in cardiovascular aging[148]. In addition to its interrelationship with ER-α-36, the GPER action also seems to interrelate with the classic ERα receptor. Several studies have demonstrated a similar effects when ERα or GPER are activated, showing an interaction between both receptors in the 17β-estradiol effect, or a common related mechanism.[149,150] A study in endometrial cancer cells reported a physical interaction between GPER and ERα. This interaction was increased by 17 β-estradiol, and prevented by the estrogen receptor antagonist ICI 182,780, suggesting that the interaction between both receptors is ligand dependent.[151]

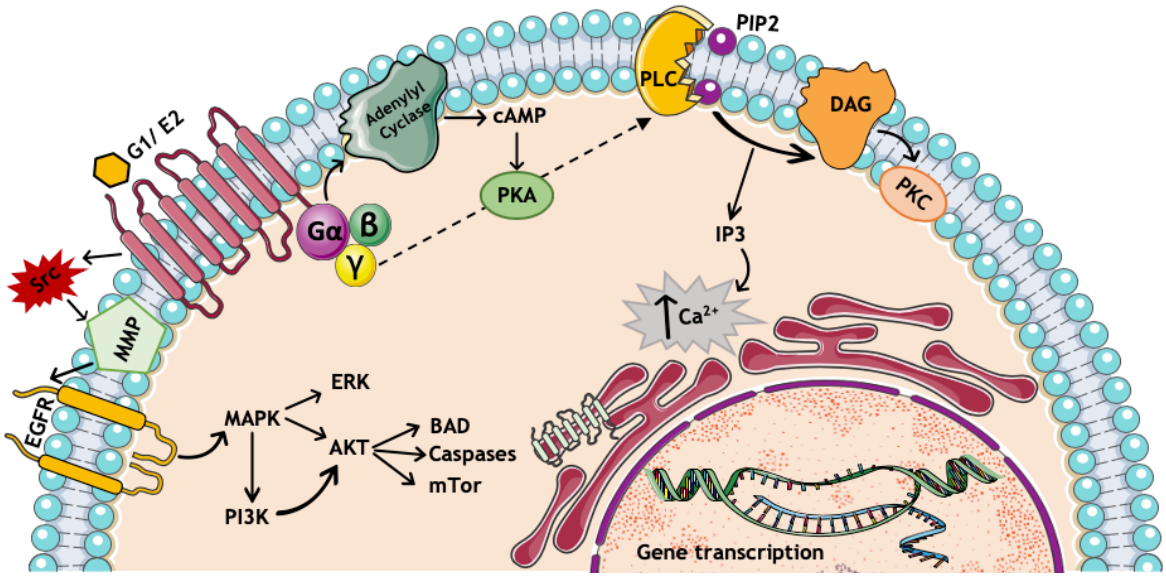

Figure 2. Schematic representation of the various signaling pathways regulated by GPER.

1.4. Neuroprotective effects mediated by GPER in PD

An important neuroprotective role of G1 was demonstrated in the MPTP PD mouse model, mediated by upregulation GDNF expression.[152] Bessa et al demonstrated that the selective activation of GPER by G1 protects mouse midbrain dopaminergic neurons against MPP^+.[152] Another study showed that G1 treatment is able to increase the concentration of dopamine, its metabolites, and also the membrane specific binding (DAT) and vesicular (VMAT) dopamine transporters in the striatum of MPTP- treated mice.[153]

The protection mediated by GPER activation in the nigrostriatal system involves also the promotion of anti-inflammatory effects[154]. G1 treatment significantly inhibits activation of microglia in SNc of MPTP-treated mice. Treatment with G1 was also able to lower the levels of proinflammatory cytokines IL-1β, TNF-α, and IL-6 in the midbrain of MPTP-treated mice, attenuating the neuroinflammation processes and the death of dopaminergic neurons characteristic of PD models.[155] On the other hand, in the Lipopolysaccharide (LPS) mouse model, GPER activation by G1 decreases IL-1 β, iNOS and CD68 mRNA levels.[154] Furthermore, GPER is implicated in regulating the expression of glutamate transporter 1 (GLT-1), in rat primary astrocytes, which may be associated with the excitotoxicity neurodegeneration in PD.[156]

Moreover, activation of GPER by the selective agonist G1 exerted a significant protective action against NMDA-induced neurotoxicity in an *in vitro* model. G1 treatment significantly attenuated the neuronal injury induced by NMDA activation with a decrease in the number of necrotic cells and apoptotic cells.[157]

1.5. 6-OHDA as a Parkinson's Disease Experimental Model

6-Hydroxydopamine (6-OHDA) is one of the most commonly used neurotoxins to induce experimental parkinsonism. This neurotoxin, a hydroxylated analogue of dopamine, causes anterograde degeneration of the nigrostriatal dopamine system when injected in the SNc. In 1968 Ungerstedt[158] showed that injection of 6-OHDA into the SNc reduced the tyrosine hydroxylase (TH) positive neurons in the injected area by 60% and resulted in the subsequent loss of the TH-positive terminals in the striatum.[159]

The neuronal damage induced by this neurotoxin is mainly due to the massive oxidative stress caused. Due to its structural similarities with dopamine, 6-OHDA has a high affinity to the dopamine transporter, which carries the toxin inside dopaminergic neurons. Once inside the neuron, 6-OHDA undergoes rapid auto-oxidation generating a high rate of free radical formation. In the mitochondria 6-OHDA inhibits the activity of the electron transport chain at the level of complex I, leading to a massive mitochondria dysfunction.[160]

Although the 6-OHDA model does not lead to the generation of Lewy-like inclusions, it has been reported to interact with α -syn inhibiting its proteasomal degradation and promoting its aggregation.[161]

Unlike other toxins used to induce PD, 6-OHDA does not pass the blood-brain barrier and therefore, is requires injection in the SNc, leading to rapid cell death, in the medial forebrain bundle, or in the STR causing slower retrograde degeneration of dopaminergic neurons.[162,160]

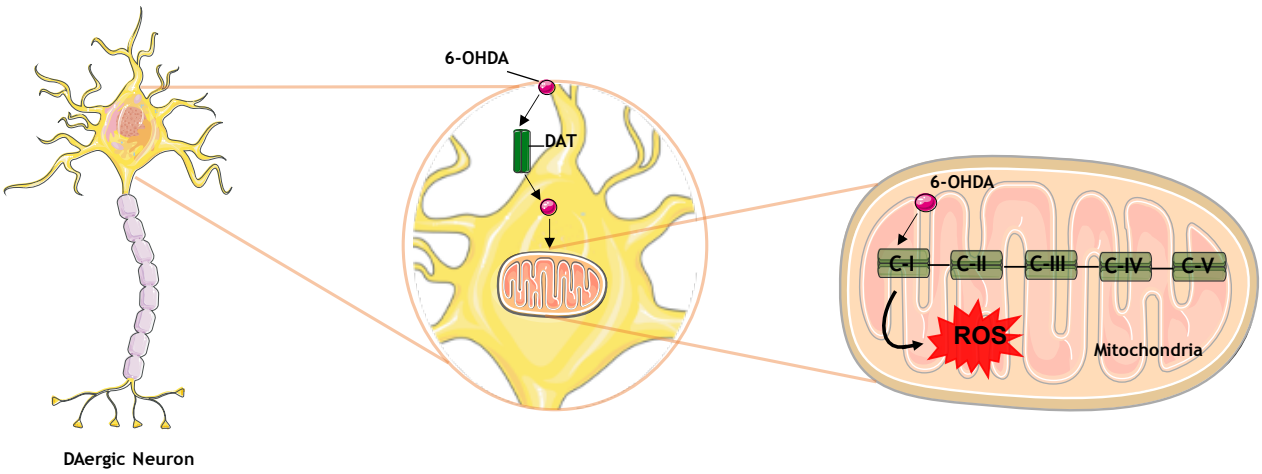

Figure 3. Schematic representation of the 6-OHDA in vivo Model.

Chapter 2
Objectives

Objectives

PD is characterized by the progressive death of dopaminergic neurons and oxidative stress is considered a major cause of this disease. On the other hand, estradiol is well-known as a neuroprotective steroid, and many of the protective effects of estradiol can be mimicked by G1. Our group has previously demonstrated that G1 can exert a neuroprotective action on dopaminergic neurons against toxins such as 6-OHDA and also against the LPS-induce injury. G1 is characterized by rapid dispersion in tissues and can reach the brain through subcutaneous delivery. However, as the nasal mucosa is a highly irrigated area and has receptors directly connected to the brain that facilitate targeting to the brain. Thus, two forms of G1 administration have been proposed in this work: subcutaneous and intranasal.

The aim of this work was to determine the effect of selective activation of GPER through G1 administration by two different approaches, subcutaneous and intranasal, on the oxidative stress induced by 6-OHDA striatal injection, and whether a putative antioxidant mechanism is capable of reducing the dopaminergic injury and the motor impairments.

Chapter 3

Materials and Methods

Materials and Methods

3.1. Animals

To perform this study male C57BL/6J mice, with approximately 3 months old and a weight between 20-27 g, were used. These animals were kept under cycles of 12h light and 12h dark and 22ºC room temperature, with water and food always available. Mice were divided into different experimental groups, handled for 5 or 7 days, and were injected on the 12th day with 6-OHDA (H4381, Sigma Aldrich) or vehicle (0.9% NaCl). The animals were then subjected to a G1 treatment (3577, Tocris Biosciences, 200 µg/kg) or to vehicle (0.3% gelatin in 0.9% NaCl)

All experiments performed on mice were conducted taking into account national ethical requirements for animal research, and in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Directive 2010/63/EU). All the procedures were previously approved by the Animal Welfare Body of the Faculty of Health Sciences, and by the National Authorities (Direcção Geral de Alimentação e Veterinária).

3.1.1. Experimental procedures

The animals were first divided into two experimental groups, intranasal and subcutaneous administration of GPER agonist. The animals subjected to subcutaneous treatment were handled only for 5 days, followed by the Rotarod training on the 6th day. All the subsequent procedures were identical to the ones used for the animals subjected to intranasal administration of G1/vehicle (Figure 4).

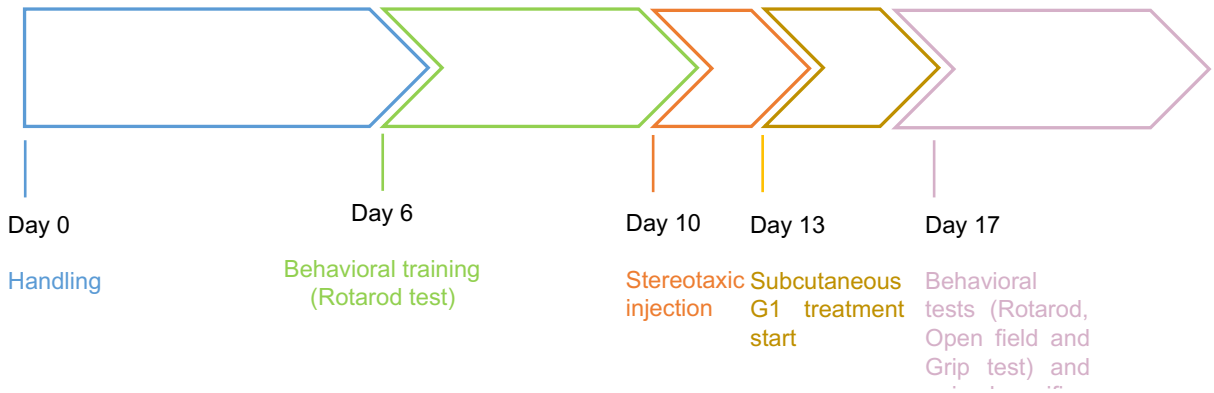

Figure 4. Schematic representation of the experimental procedure used for the G1 treatment, by subcutaneous administration in the 6-OHDA model.

The intranasal treated animals were submitted to 7 days of handling, followed, by the Rotarod training, in the 8th day. The stereotaxic injection was performed on the 12th day of the experiment, and treatment with G1 or the vehicle started on the 15th day. The last step of the experiment took place on the 19th day, with behavioral tests (Rotarod, Open Field and Grip test) and animal sacrifice (Figure 5).

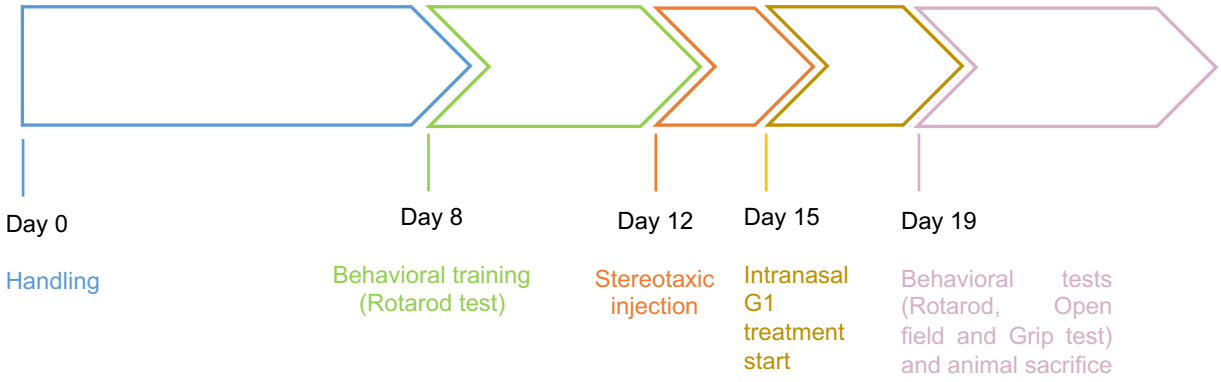

Figure 5. Schematic representation of the experimental procedure used for the G1 treatment, by intranasal delivery, in the 6-OHDA model.

3.1.2. The effect of striatum 6-OHDA injection on the body weight change in mice

As a way to control the wellbeing of the animals and of evaluating the continuity of the experimental procedure, the weight of the animals was monitored during all the experiment (Figure 6). Although the stereotaxic injection procedure induced a decrease in the animals' weight in the days following the procedure, all animals regained their weight.

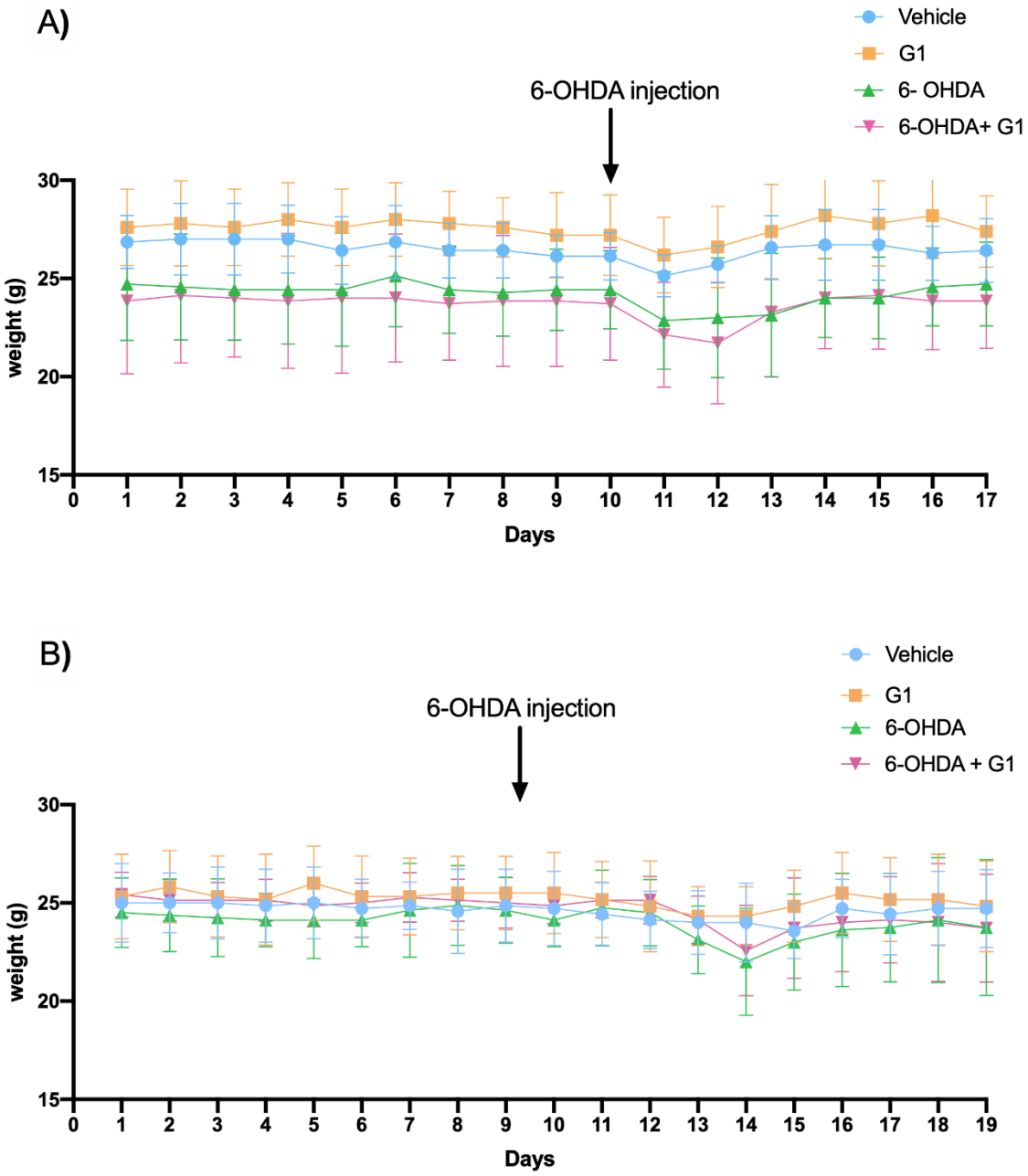

Figure 6. Changes of mice body weight during the experimental procedure. The graph shows weight changes in the group subjected to subcutaneous (A) and intranasal (B) injection. Each value represents the mean of 5-7 animals from each experimental group.

3.2. GPER agonist administration

3.2.1. G1 Subcutaneous administration

For the subcutaneous administration animals were immobilized and the needle was inserted at a 45º angle at the base of the skin fold created in on the back of the animal. The administration of the substance was always done in a stable, constant and firm way. The dose subcutaneously administered per animal was 6 µg G1/ 100 µl vehicle animal.

3.2.2. G1 intranasal administration

Intranasal administration represents a more delicate form of administration. The animals were immobilized, creating a bend in their dorsal part and are then turned belly up so that it is possible for the administrator to see the nasal cavities. The immobilization technique is very important, so that they are not able to turn around. Once immobile, the substance was administered in a volume of 5 µl in each nostril, pouring small drops with a micropipette. In this step, it is very important to pour a small volume at a time, to ensure that everything is absorbed and to prevent the animals from sneezing. In total, a dose of 6 µg G1/ 10 µl Vehicle/ animal was administered.

All in vivo techniques were performed with the utmost care in order to introduce as little stress as possible to the animal. In this way, procedures were always performed in the same way, at the same time and by the same person. To avoid the stress responses of the animal, previous training/handling was performed as mentioned. For the subcutaneous administration, the training was done with a pen. The training consisted of immobilizing the animal, bending and placing the tip of the pen where we should put the needle to administer the substance. For intranasal administration, the training was done with NaCl. Thus, the animals were immobilized, and 5 µl of NaCl/ 30g animal in each nostril were applied.

3.3. Behavioral Evaluation

3.3.1. Open field test

This test is considered as an exploratory which is based on the animals' willingness to explore new spaces. The day of the test should be the first day that the mice is in contact with the enclosure. A healthy animal will move around to explore the place, with frequent rises (z axis movements).

The open field test is performed in the last day of the procedures. In order for the animal to get used to the environmental conditions of the room the mice were placed in the room where the test was performed 30 minutes before starting the test. This test was done in the same period of the day for all animals. At the beginning of the test the animal was placed in the center of the arena and movements in the horizontal and vertical plan were registered with the ActiMot2 system (TSE Systems) for 10 minutes.

3.3.2. Rotarod test

The Rotarod test consists of a resistance training based on an accelerating rotating rod. Some of the parameters evaluated with this test include balance, strength and motor coordination of the mice. The test was performed daily for 4 days before the stereotaxic injection to allow the animal to get used to the procedure. Only the results from the last day were considered. Every day, at the same time the mice were placed on the device (cat. 47600; UGO basile) at an initial speed of 4 rpm that increased to 40 rpm in a period of 5 minutes. The test was done before stereotaxic surgery and after surgery and G1 administration.

3.3.3. Grip test

This test consists of placing the mouse on a grid, turning the grid upside down at a considerable height and recording the time that each animal can stand without falling. An animal without motor impairments should hold on for a longer period of time.

3.4. Stereotaxic surgery and animal sacrifice

The stereotaxic surgery was performed on the 10th day of the experiment. 6-OHDA or the vehicle (0.9% NaCl) were injected under ketamine (196 mg/Kg) and xylazine (13 mg/kg) anesthesia. About 30 minutes after the first dose, a second dose of anesthesia was given (40.82 mg ketamine and 4.02 mg xylazine). 6-OHDA (10 µg) was dissolved in 0.9% NaCl with 0.2% ascorbic acid and injected with a 10 µl Hamilton syringe and a Stoelting Quintessential Stereotaxic injector in the right side of the striatum using the following coordinates: 0.4 mm posterior, 1.8 mm/ -1.8 mm lateral to bregma and -3 mm ventral to the bregma, according to the Mouse brain atlas[163]. The injection occurred for 10 minutes at a rate of 0.2 µl/min, and then the needle was left for further 5 minutes after the injection to allow the liquid to diffuse. The needle was then slowly retracted from the brain.
The work involved four experimental conditions: Control, G1, 6-OHDA and, 6-OHDA + G1 (table 1).

Table 1. Description of the experimental groups.

	Stereotaxic injection		**Treatment**	
Experimental conditions	**Left Side STR**	**Right side STR**	**Intranasal**	**Subcutaneous**
Control	-	0.9% NaCl	G1 vehicle (0.3% gelatin in 0.9% NaCl solution)	
G1	-	0.9% NaCl	G1(6 µg G1/30g animal weight)	
6-OHDA	6-OHDA vehicle (0.9% NaCl)	6-OHDA (10 µg)	G1 (0.3% gelatin in 0.9% NaCl solution)	
6-OHDA + G1	6-OHDA vehicle (0.9% NaCl)	6-OHDA (10 µg)	G1(6 µg G1/30g animal weight)	

The animals that were not injected with 6-OHDA were injected with 0.9% NaCl. Two types of G1 treatment were performed: intranasal (6 µg G1/ 10 µl Vehicle/ 30g animal weight) and subcutaneous (6 µg G1/ 100 µl Vehicle/ 30g animal weight). In both control and 6-OHDA groups only G1 vehicle was administered, which consisted of 0.3% gelatin dissolved in 0.9% NaCl solution. G1 and 6-OHDA + G1 groups were injected with 6 µg G1/30g animal weight, dissolved in 0.3% gelatin in 0.9% NaCl solution.
Seven days after the stereotaxic surgery, animals were sacrificed by cervical dislocation, and the brains were stored at -80ºC. For the immunohistochemistry analysis the animals under ketamine and xylazine anesthesia were transcardially perfused with saline and 4%

paraformaldehyde (PFA). The animals were decapitated, and the brains removed and placed at 4% PFA for 1 day. Later, they were placed in PBS in a cryoprotective solution (30% sucrose in PBS) for approximately 2 days. Finally, the brains were pre-frozen in liquid nitrogen and, after that, frozen at -80ºC.

3.5. Brain Slicing Procedure

The brains maintained at -80ºC were transferred to a freezer at -20ºC for 30 minutes before being transferred to the cryostat (Leica CM3050) chamber, at a temperature of -22ºC. Subsequently, the brains were positioned in the object holder at -20ºC temperature and were sliced in 35 µm thick coronal sections. Slices corresponding to the STR and midbrain were collected to 24 wells plates (Orange scientific). All coronal sections were stored in antifreeze solution (62.5 mM sodium phosphate buffer (pH 7.4), 18.75% sucrose and ethylene glycol), at -20ºC.

3.6. Immunohistochemistry

In order to initiate the Immunohistochemistry (IHC) procedure, brain sections were firstly transferred from the antifreeze solution to PBS containing 0.1% Tween (PBS-T) and then, were permeabilized with PBS with 1% Triton X-100, at room temperature. To reduce non-specific binding, the brain sections were incubated with PBS with 10% Fetal Bovine Serum (FBS) for 2 hours. All antibodies specified in table 2 were diluted in PBS-T containing 10% FBS. Incubation with the primary antibodies was done at 4ºC for 48 hours. Next, the sections were washed with PBS-T three times for 15 min, and then incubated with the secondary antibodies for 18 hours at room temperature. After three 15 min washes with PBS-T, the sections were incubated with 2 µM Hoechst (33342, Invitrogen), diluted in PBS-T, for 10 min at room temperature. Finally, the sections were mounted with DAKO medium in Superfrost slides. The slides and coverslips were sealed using nail polish. Fluorescent images from the sections were obtained with a confocal microscope (LSM 710, Zeiss) with 20x and 40x objectives. Images corresponding to the SNc regions were captured.

Table 2. Description of antibodies used in Immunohistochemistry Assay.

Protein	Primary Antibody	Dilution	Supplier	Secondary Antibody	Dilution Factor	Supplier
TH	Rabbit Polyclonal Anti-TH	(1:1000)	Santa Cruz Biotechnology	Goat Anti-Rabbit conjugated to Alexa 546	(1:1000)	Invitrogen
8-OxoG	Mouse Monoclonal Anti-8-OxoG	(1:400)	Chemicon	Goat Anti-Mouse conjugated to Alexa 488	(1:1000)	Invitrogen

3.7. Total RNA extraction

40 µl of PBS 1x were added to the eppendorf containing the tissue. In order to facilitate the disruption of the tissue, the tissue was homogenized with a pestle against the walls of the Eppendorf and 25 µl are transferred to a new eppendorf. Then a fast centrifugation at 14,100 x g was done (mini spin plus, eppendorf) and the supernatant was collected and discarded. The extraction of total RNA started with the addition of Trizol (TRI, 2302700 5prime). This compound consists of a single-phase solution of phenol and guanidinium isothiocyanate responsible not only for cell disruption but also for maintaining the integrity of the RNA. 175 µl were added and incubated 5 minutes at room temperature. After that, 35 µl of chloroform was added to promote the separation of three phases, a lower organic phase containing proteins, an intermediate phase with DNA and an upper aqueous phase containing RNA. The entire content was mixed by inversion and incubated for 10 minutes at room temperature. Subsequently, the samples were centrifugated at 12,000 g for 15 minutes (Mikro 200R, Hettich Zentrifugen), the aqueous phase was recovered t a new Eppendorf to which 87.5 µl isopropanol was added and the sample was mixed by vigorous inversion. After a 10 minutes incubation at room temperature, the sample was centrifugated at 12,000 g for 10 minutes (Mikro 200R, Hettich Zentrifugen), the supernatant was rejected, and the pellet was washed with 87.5 µl ethanol 75% in water 0.01% diethylpyrocarbonate (DEPC) at -20ºC to remove impurities. After a final centrifugation at 7,500 g for 5 minutes, the excess ethanol is removed, the pellet is rehydrated with DEPC water and finally the total RNA samples are stored at -80ºC.

3.8. Total RNA quantification

Total RNA concentration was determined by spectroscopy in a UV/ Vis Nanophotometer TM spectrophotometer (Implen) at 260 nm. The purity of the RNA was analyzed through the ratio (A260/280), ensuring that it was in the range 1.8 - 2.0 since a ratio below 1.8 indicates the presence of proteins while a ratio above 2.0 indicates DNA contamination.

3.9. RNA integrity

The integrity of RNA was confirmed by the presence of two evident bands in an electrophoresis gel in which the upper ribosomal band (28S in eukaryotic cells) should be about twice the intensity of the lower band (18S in eukaryotic cells). This gel consists of 1% agarose with 0.05% Green safe, a nucleic acid intercalator. The gel was then visualized on an UVITEC transilluminator (UVitec Cambridge, United Kingdom).

3.10. cDNA synthesis

cDNA synthesis was performed using reverse transcriptase. cDNA was synthesized from 1 µg total RNA and two mixtures were prepared with a volume sufficient to n+1 reactions. Thus, to 1 µg of total RNA was added 1 µl of Random primers (50 ng/ µl, MB12901 Nzytech),1 µl of DNTPs (10 mM, R0181 Thermo Scientific) and 17 µl sterile nuclease-free water. This mixture was incubated in the thermal cycler (T100 ™ Thermal Cycler Biorad) at 65ºC for 5 minutes. After that, 2 µl of 10x buffer (Nzytech) and 1 µl Moloney Murine Leukemia Virus reverse transcriptase (20000U M-MuLV RT, Nzytech) was added to each sample. This was followed by a 50-minute incubation at 37°C and a final 15-minute incubation at 70°C to inactivate the enzyme.

3.11. Conventional PCR

The conventional polymerase chain reaction (PCR) allows us to verify the detection of the desired genes in the samples used in the study. A mix consisting of 2.5 µl 10x Buffer, 0.625 µl Taq DNAPolymerase (EP0702, Thermo Fisher Scientific), 0.5µl DNTPs (10mM, R0181), 0.75µl forward and reverse primer (10mM), and 18.87 µl to obtain a final volume of 24 µl

was prepared for each sample. One µl of cDNA was added for each reaction, except for the negative control. To amplify the fragments Taq DNA polymerase was used. The protocol consisted of heating the samples to 95ºC for 3 minutes followed by 38 cycles of 95ºC for 30 seconds. After that, annealing of the primers for 30 seconds, 1 minute at 72ºC and, to finalize, 5 minutes at 72ºC. The products obtained by PCR were run on a 0.2% agarose gel with 0.05% Green Safe. In the first well of the agarose gel, 5 µl of NZYDNA Ladder VI (MB08901, Nzytech) was deposited, and in the remaining ones, 9 µl of each PCR product and 1 µl of loading buffer 10x was added.

Table 3. Description of primers used in PCR.

Gene	Primers (5'-3')	Fragment size (bp)
SOD1	FW 5' CCACTGCAGGACCTCATTTT 3' RV 5' CACCTTTGCCCAAGTCATCT 3'	197
CAT	FW 5' ACATGGTCTGGGACTTCTGG 3' RV 5' CAAGTTTTTGATGCCCTGGT 3'	216
GPX4	FW 5' CCTTCCCCTGCAACCAGTTT 3' RV 5' CCACGCAGCCGTTCTTATCA 3'	234
NOX1	FW 5' GGAGTTGCAGGCATCCTCAT 3' RV 5' TTCCATGCTAAAGCCTCGCT 3'	197
Nrf2	FW 5' ATGATGGACTTGGAGTTGCCA 3' RV 5' GCTCATAGTCCTTCTGTCGC 3'	142

3.12. Real time PCR (RT-PCR)

RT-PCR was used to evaluate the expression of antioxidant enzymes such as SOD1, CAT, GPX4 and NRF2 in samples from mice brains. The first step was to test the efficiency of the primers. For this, 4 dilutions were used for each primer (1:1, 1:5, 1:25 1:125). For each reaction 1 µl of cDNA was added to a mix containing 10 µl of Luminaris HiGreen Fluorescein qPCR Master Mix (K0983, Thermo Fisher Scientific), 0.3 µM (SOD1, CAT and GPX4) or 0.15 µM (NRF2) of each primer and sterile water. To normalize the levels of gene expression the Cyclophilin A gene (CyPA) was used as a housekeeping gene. Real time PCR reactions were settled according to table 4 in a thermocycler CFX Connect™ (Real-Time System, Bio-Rad) and mRNA expression was determined using the cycle time values normalized to values of housekeeping gene. The results are expressed as $2^{-\Delta\Delta CT}$ relative to control based on the Pfaffl method.

Table 4. Description of the experimental protocol used in RT-PCR.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Cycles
SOD1/CyPA	95 ºC 3 min	95º C 30 s	55º C 30 s	72º C 1 min	38x
CAT/CyPA	95 ºC 3 min	95º C 30 s	59ºC 30 s	72º C 1 min	38x
GPX4/CyPA	95 ºC 3 min	95º C 30 s	61 ºC 30 s	72º C 1 min	38x
NRF2/CyPA	95 ºC 3 min	95º C 30 s	61 ºC 30 s	72º C 1 min	38x

3.13. Statistical Analysis

Data are expressed as percentages of values obtained in control conditions and are presented as mean ± Standard Error of the Mean (S.E.M). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's test. Values of $p<0.05$ were considered significant and all the procedures were performed using GraphPad Prism 8 (GraphPad Software, In.). In the evaluation of the relative levels of mRNA for the different enzymes, samples from SNc were standardized to left SNc (contralesional side). Samples from STR were also standardized to left STR (contralesional side).

Chapter 4

Results

Results

In order to assess whether the injected toxin had an effect on the motor and non-motor behavior of the animals, we used behavioral tests such as Grip test, Rotarod and Open field test.

4.1. Behavioral assessment

The behavioral assessment was done with the Rotarod Test, Open Field Test, and Grip Test. Rotarod Test aims to evaluate balance and coordination of the mice. The Open Field test is used to analyse both anxiety and exploratory behavior and relies on the tendency of the animals to explore the arena, and to elevate in order to explore the surroundings.
Assessment of motor functions was obtained from behavioral tests performed in the 13th and 15th days of subcutaneous and intranasal administration, respectively.
The grip test measures the maximum isometric strength of the hands and forearm muscles and is classified as a general strength test. In animals injected subcutaneously the time the animals remain suspended was similar in the different experimental conditions and, contrary to what might be expected, the time held in the grip test did not decrease in animals injected with 6-OHDA.

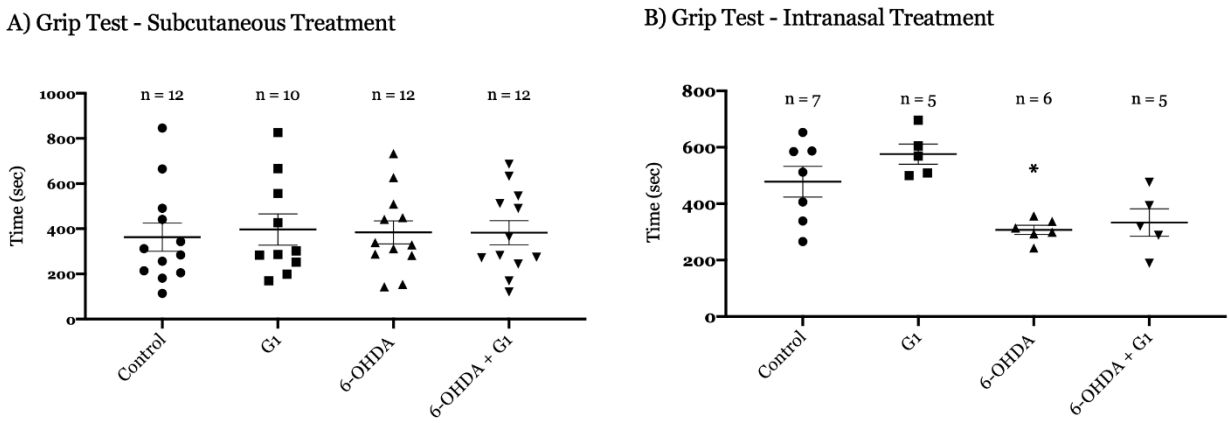

Figure 7. Effect of GPER activation by G1 on the 6-OHDA-induced motor impairments assessed by Grip Test in Subcutaneous (A) and Intranasal (B) treated animals. Mice were subjected to stereotaxic injection of vehicle or 6-OHDA and were later treated with G1 or vehicle. At the end of the treatment, the animals were subjected to behavioral tests. Data represents the mean ± standard error of the mean (SEM) of data from 5 to 7 animals (Intranasal treatment) and 10 to 12 animals (Subcutaneous treatment). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. (*$p < 0.05$ compared to Control group).

In what concerns the intranasal administration of G1, animals treated with G1 presented a non-statistically significant increase (approximately 20%) on the time that they remain suspended, when compared to the control group. The 6-OHDA-treated mice exhibited a decrease of 35.78% in the time they remain suspended when compared to the control group. The G1 administration (6-OHDA + G1 group) was not significantly by 6-OHDA.

The Rotarod test, measures the time that the mice stand on the rotating rod and is used to assess motor coordination, balance and global physical condition.

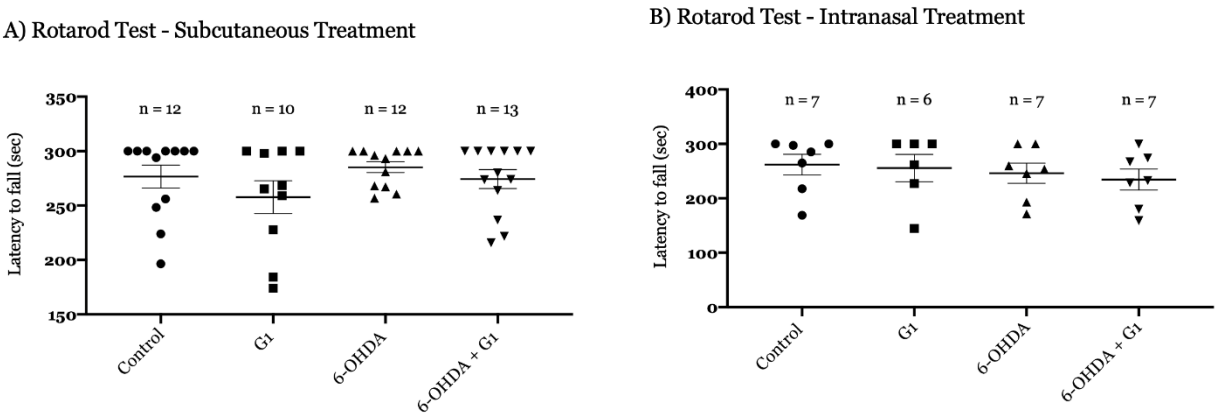

Figure 8. Effect of GPER activation on the 6-OHDA-induced motor impairments assessed by Rotarod Test in Subcutaneous (A) and Intranasal (B) treated animals. The animals were subjected to stereotaxic injection of vehicle or 6-OHDA and were later treated with G1 or vehicle. Data represents mean ± standard error of the mean (SEM) of 6 -13 animals from different experimental conditions.

6-OHDA injection and G1 subcutaneous or intranasal administration had no effect on the latency of the animals to fall (Figure 8).

The open field test is considered an exploratory test and gives information about the exploratory abilities of animals as well as of their motor activity and anxiety. The exploration of the arena was recorded during 10 minutes (Figure 9, 10 and 11).

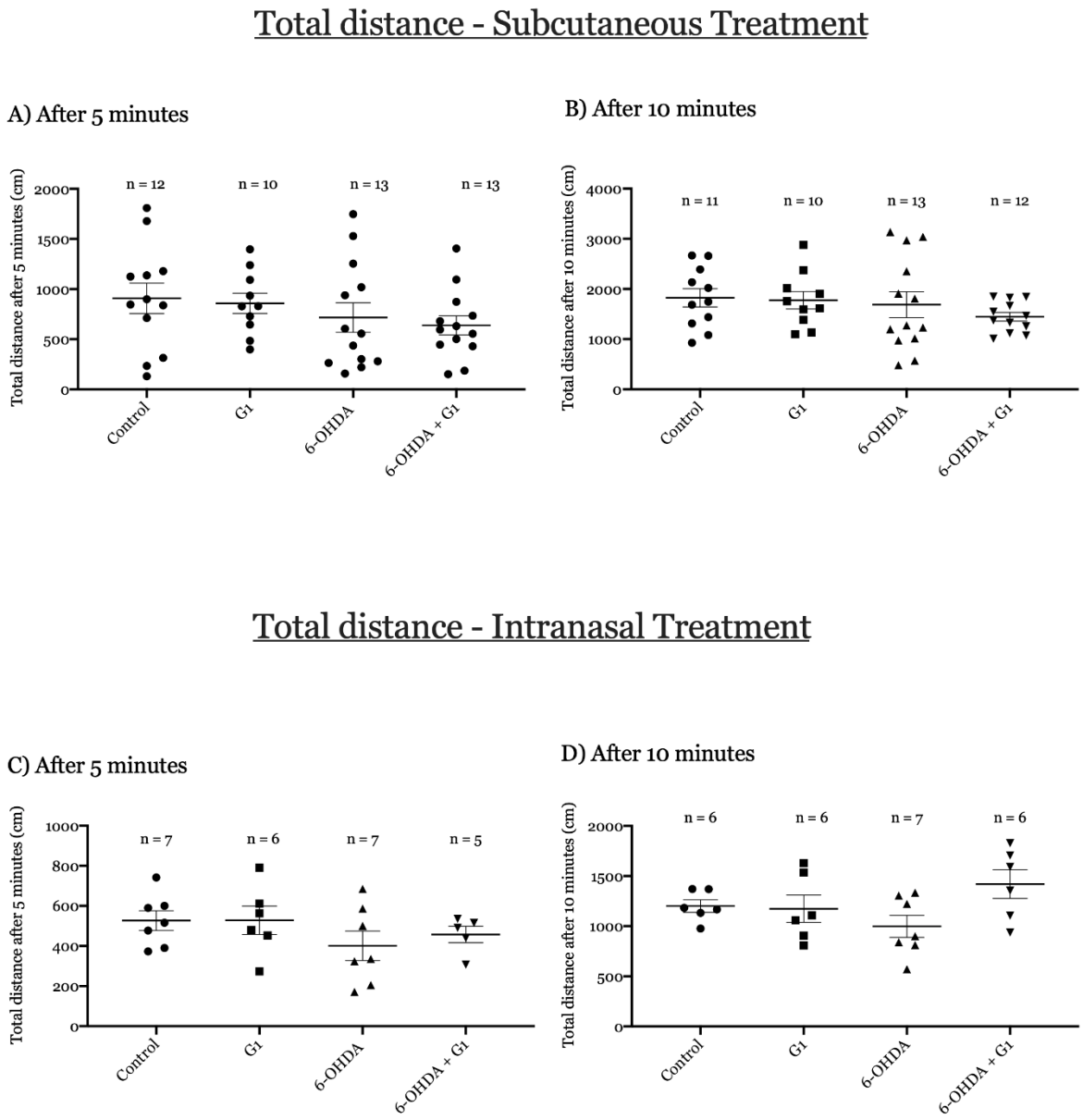

Figure 9. GPER activation on the 6-OHDA injected mice was unable to induce motor impairments in total distance travelled assessed by Open Field Test in Subcutaneously (A, B) and in Intranasally (C, D) treated animals. The animals were subjected to a stereotaxic injection of vehicle or 6-OHDA and were later treated with G1 or vehicle. At the end of the treatment, the animals were subjected to Open field test. In this test, different parameters were studied such as the total distance traveled during 5 (A, C) and 10 minutes (B, D). Data represents the mean ± standard error of the mean (SEM) of 5-7 animals as indicated in the graph for each experimental condition.

Analysis of the total distance traveled by the animals of the different experimental conditions, either treated subcutaneously (Figure 9 A, B) or by intranasal delivery of G1 (Figure 9 C, D) did not show any significant difference, either at 5 or 10 minutes.

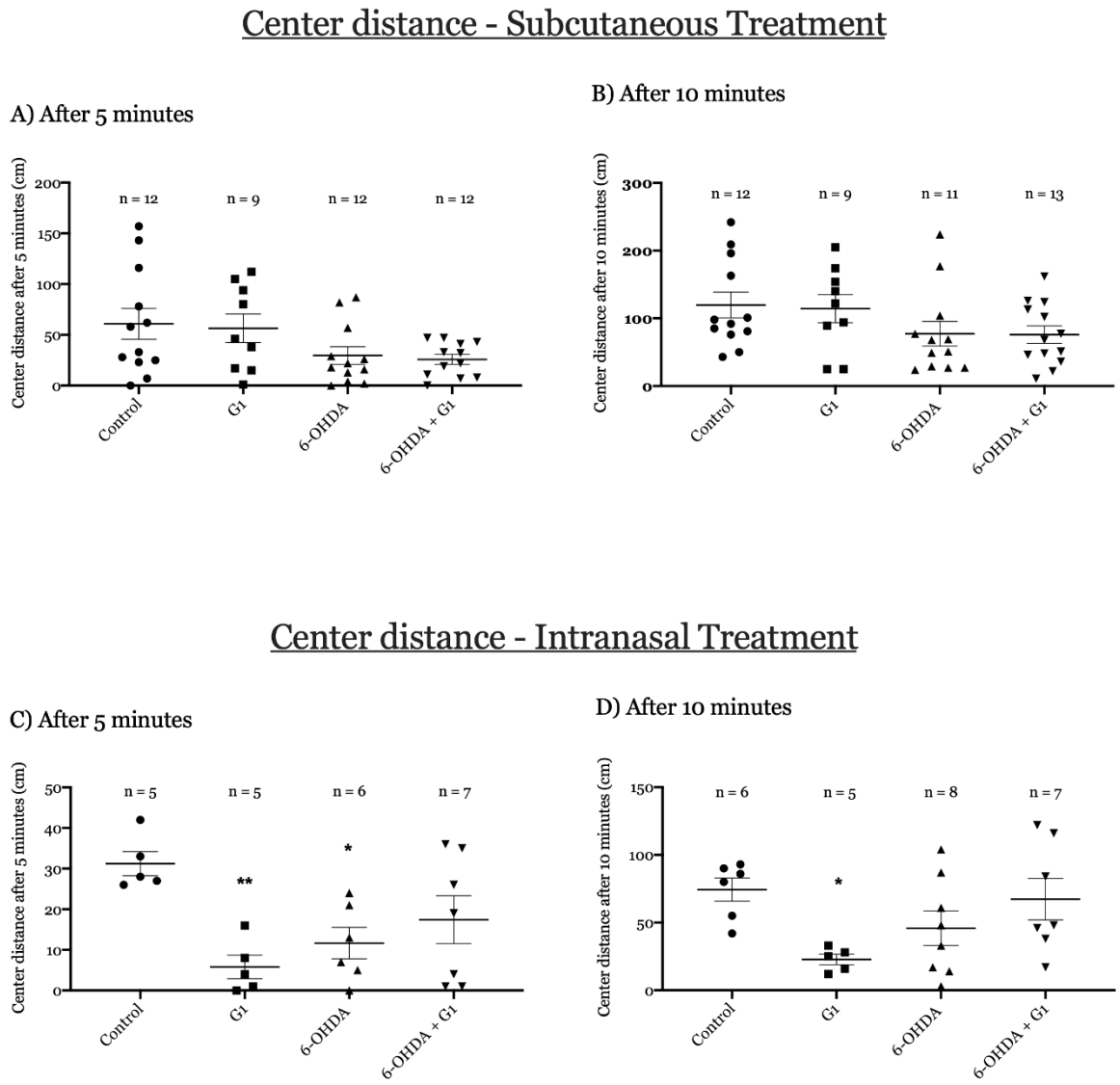

Figure 10. Effect of GPER activation on the 6-OHDA induced motor impairments evaluated by the distance travelled in the center of the arena.
A, B - Subcutaneous administration of G1; C, D- Intranasal administration of G1. The animals were subjected to a stereotaxic injection of vehicle or 6-OHDA and were later treated with G1 or vehicle. At the end of the treatment, the animals were subjected to the Open field test. The distance travelled in the center of the arena was recorded during 5 (A, C) and 10 minutes (B, D). Data represents the mean ± standard error of the mean (SEM) of 5-13 animals as indicated in the graph for each experimental condition. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test (*$p < 0.05$, **$p < 0.001$ compared to Control group).

In what concerns the distance travelled specifically in the center of the arena, which is used to measuring animal anxiety, 6-OHDA injection induced a decrease in the distance travelled in the center of the arena, 51.22% decrease at 5 min (Figure 10 A) and 35.51% decrease at 10 minutes (Figure 10 B).

In what concerns the animals subjected to intranasal administration (Figure 10 C, D), there was a decrease of 81.41% and of 69.33% in the distance travelled in the center of the arena

for the G1 group at 5 and 10 minutes, respectively. In addition, there was a decrease of 62.50% in the 6-OHDA group at 5 minutes (Figure 10 C), and a 38.28% decrease at 10 minutes (Figure 10 D). Interestingly the 6-OHDA + G1 group seemed to recover, showing an increase in the time spent in the center of the arena when comparing with the 6-OHDA group, both at 5 and 10 minutes.

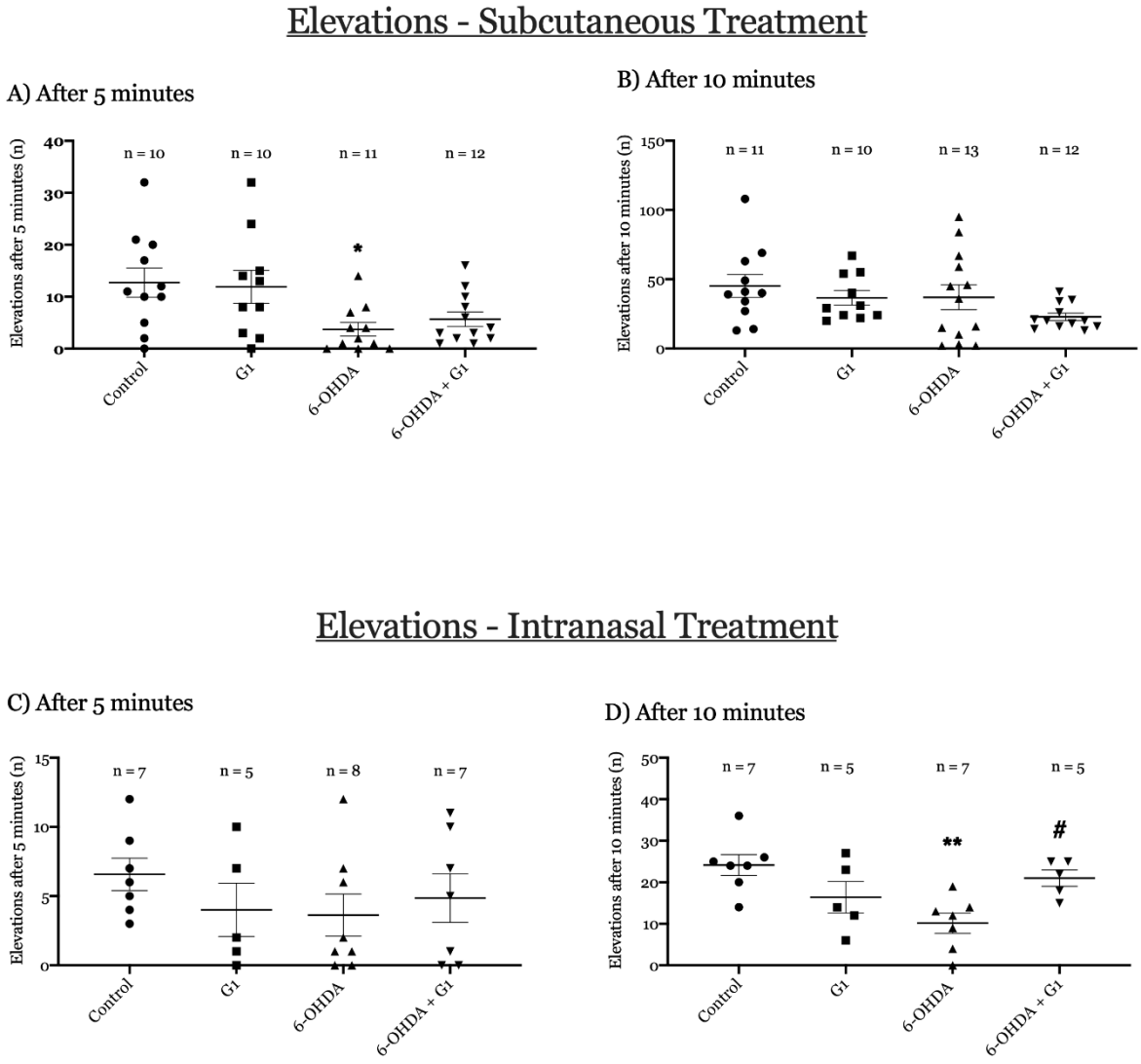

Figure 11. Effect of GPER activation on the 6-OHDA-induced motor impairments in the number of elevations assessed in Open Field Test in Subcutaneous (A, B) and in Intranasal (C, D) treated animals. The animals were subjected to a stereotaxic injection of vehicle or 6-OHDA and were later treated with G1 or vehicle. At the end of the treatment, the animals were subjected to Open field test. In this test, of the vertical elevations were registered during 5 (A, C) and 10 minutes (B, D). Data represents the mean ± standard error of the mean (SEM) of 5-13 animals as indicated in the graph for each experimental condition. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. (*$p < 0.05$, **$p < 0.001$ compared to Control group and #$p < 0.05$ compared to 6-OHDA group).

The number of vertical elevations in the Open field arena is indicative of the exploratory behavior of the mice. This test informs about the interest of mice in exploring the surroundings and is characteristic of a non-anxious behavior. The injection of 6-OHDA in

the animals treated subcutaneously with G1 induced a decrease of 70.70% in the vertical elevations registered during 5 minutes (Figure 11 A).

Regarding the intranasal treatment, no changes were observed between the different experimental groups in the first 5 minutes. However, at 10 minutes, the 6-OHDA group presented a 58.0% decrease in the number of elevations when compared to the control group (Figure 11 D). Finally, the 6-OHDA + G1 group showed a significant increase after 10 minutes (107%, D) comparing to the 6-OHDA group.

4.2. Effect of GPER activation on the expression of antioxidant enzymes

As mentioned in the previous chapters, 17β-Estradiol play a protective role in the nervous system. Therefore, it seemed important to evaluate how one of its receptors contributes to this protection. Knowing that the brain of Parkinson's patients presents an environment of elevated oxidative stress and a decrease in their antioxidant defences, we have evaluated how the expression of the antioxidant defences is affected by the specific activation of GPER In this way, we induced GPER receptor activation by G1 and evaluated the expression of enzymes relevant to the control of oxidative stress (SOD1, CAT, GPX4), and of a transcription factor relevant to the control of antioxidant defenses (Nrf2), in control conditions and also upon injection of 6-OHDA. The mRNA levels of these enzymes were analyzed in the SNc and in STR.

Unlike the other isoforms, SOD1 is located in the cytoplasm and appears to be the main isoform involved in controlling oxidative stress. *In vivo* and *in vitro* studies have shown that SOD1 expression is induced when rats or wild type cells (WT) are exposed to ROS.[164]
Regarding glutathione peroxidase, while GPX1 reduces inorganic hydrogen peroxide, GPX4 is a phospholipid hydroperoxidase that protects cells against membrane lipid peroxidation.[165]

In this sense, we use the RT-PCR to evaluate the expression levels of Nrf2 and the antioxidant enzymes. The main objective was to evaluate how the activation of the GPER receptor altered the levels of antioxidant enzymes and whether it affected the global levels of oxidative stress.

4.2.1. Relative SOD1 mRNA levels

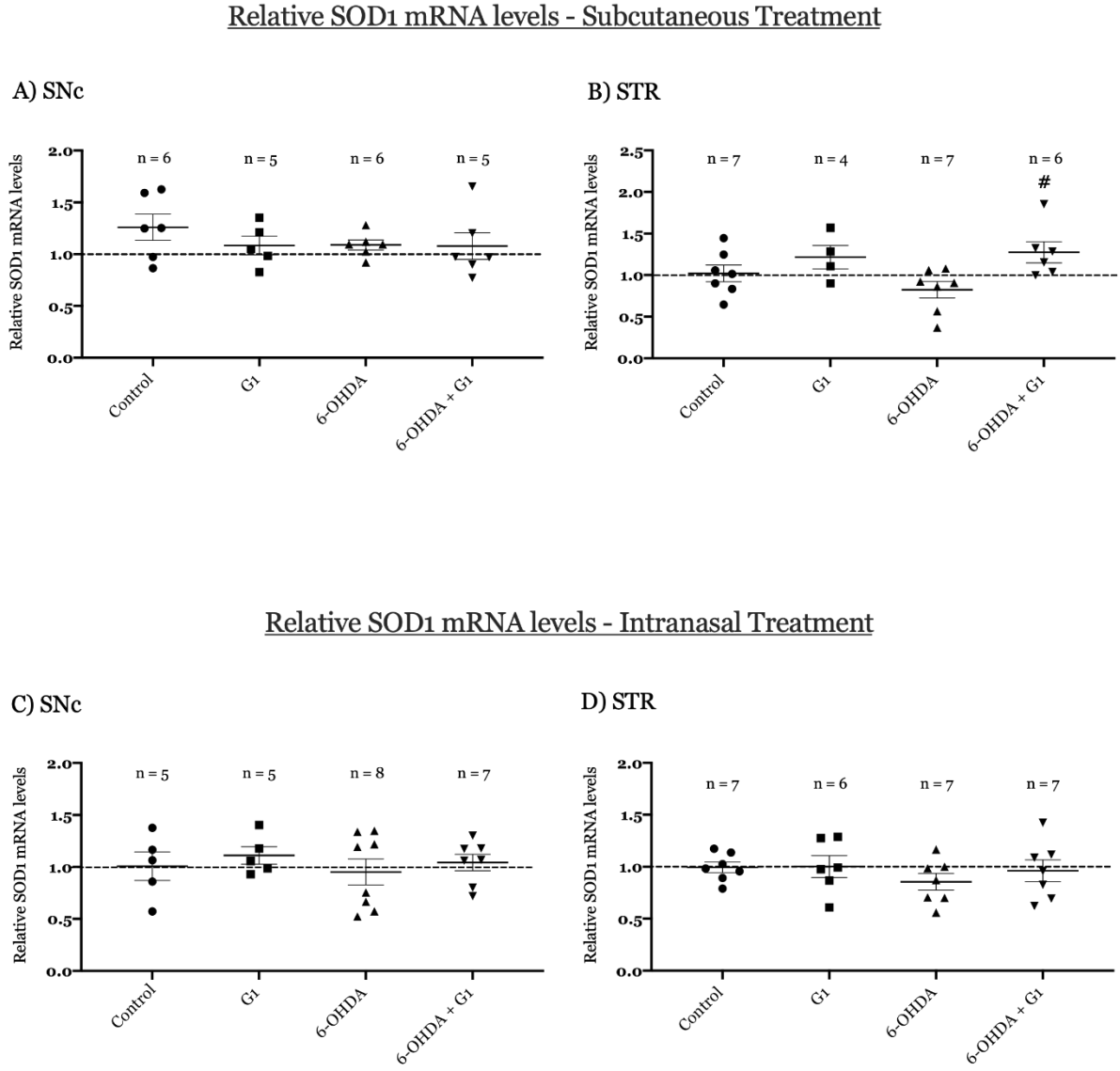

Figure 12. Effect of GPER activation on mRNA levels of superoxide dismutase 1 in mice subjected to Subcutaneous (A, B) or Intranasal (C, D) administration of G1. The animals were subjected to a stereotaxic injection of vehicle or 6-OHDA and were later treated with G1 or vehicle. At the end of the treatment, the animals were sacrificed and the region corresponding to the substantia nigra (A, C) or to the striatum (B, D) was collected. The values were normalized to the contralesional side (left side). Data represents the mean ± standard error of the mean (SEM) of 5-8 animals as indicated in the graphs. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. (#$p < 0.05$ compared to 6-OHDA group).

We analysed the expression levels of SOD1, the enzyme that catalyses the dismutation of superoxide into oxygen and peroxide. The data from the SNc and STR tissues shows that SOD1 mRNA levels were not significantly affected by 6-OHDA injection or by the exposure to the GPER agonists, either when administered subcutaneously or intranasally (Figure 12).

4.2.2. Relative CAT mRNA levels

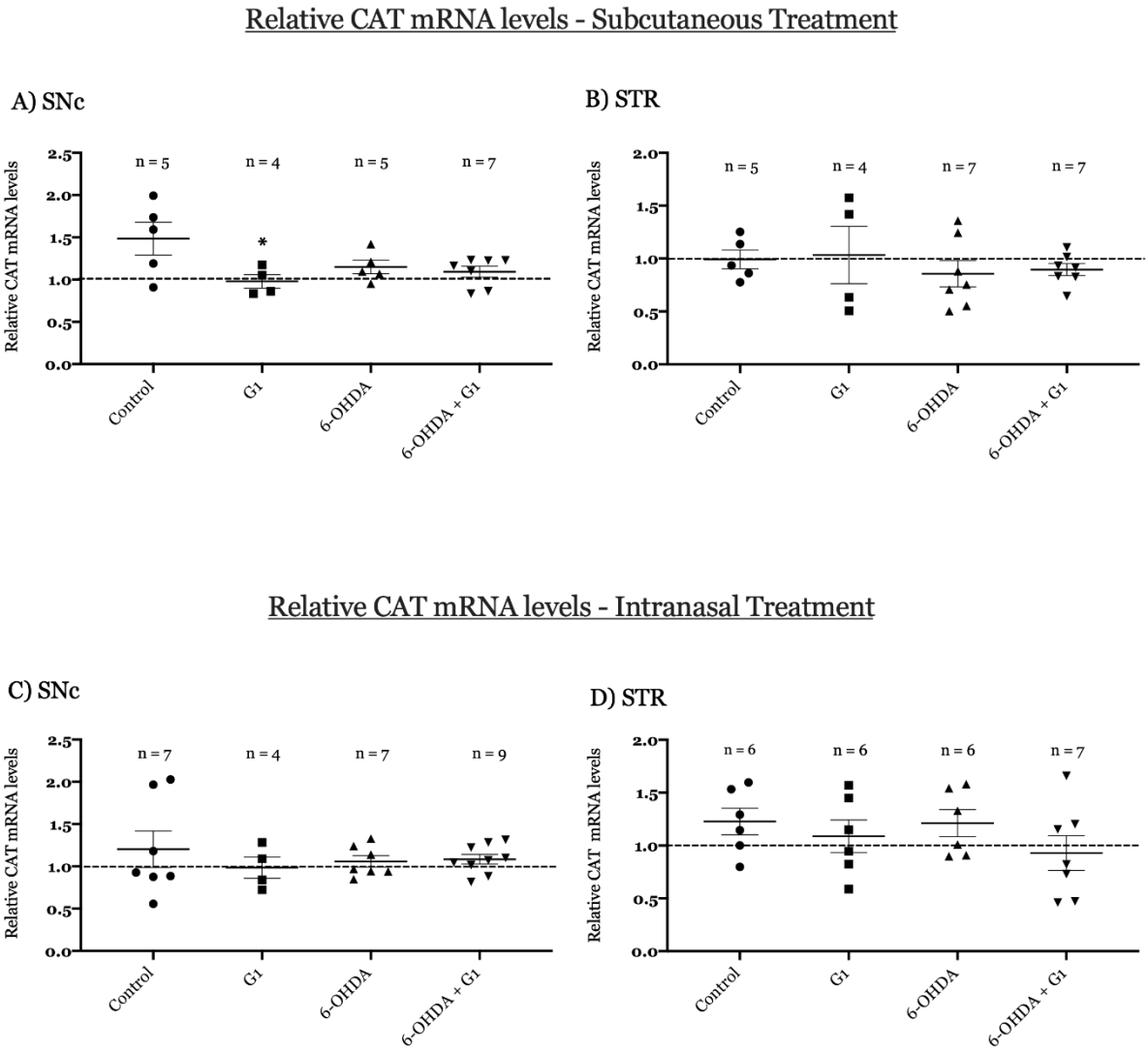

Figure 13. Effect of GPER activation on relative mRNA levels of catalase in G1 Subcutaneous (A, B) and Intranasal (C, D) treated mice. The animals were subjected to a stereotaxic injection of vehicle or 6-OHDA and were later treated with G1 or vehicle. At the end of the treatment, the animals were sacrificed and the region corresponding to the substantia nigra was collected. The values of the nigra were normalized to the left nigra (A, C) and the values of the striatum were normalized to the left striatum (B, D). Data represents the mean ± standard error of the mean (SEM) of five to nine animals from different experimental conditions. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. (#$p < 0.05$ compared to 6-OHDA group). Data represents the mean ± standard error of the mean (SEM) of five to thirteen animals from different experimental conditions. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. (*$p < 0.05$ compared to Control group.

Concerning catalase, the enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen, data from the SNc show that injection with 6-OHDA induced a decrease, although not statistically significant, of 22.6% in the mRNA levels of the enzyme (Figure 13 A). Subcutaneous treatment with G1 also induced a decrease of 34.0% in CAT mRNA levels. CAT mRNA levels in animals injected with 6-OHDA and subcutaneously

treated with G1 were similar to the levels obtained in animals injected with 6-OHDA and not subjected to G1 administration (Figure 13 A).

CAT mRNA levels in the STR of animals subjected to subcutaneous treatment with G1 was not affected by any of the treatments. Similarly, CAT mRNA levels in mice subjected to intranasal administration of G1 were also not affected by 6-OHDA injection or by the G1 treatment (Figure 13 C and D).

4.2.3. Relative GPX4 mRNA levels

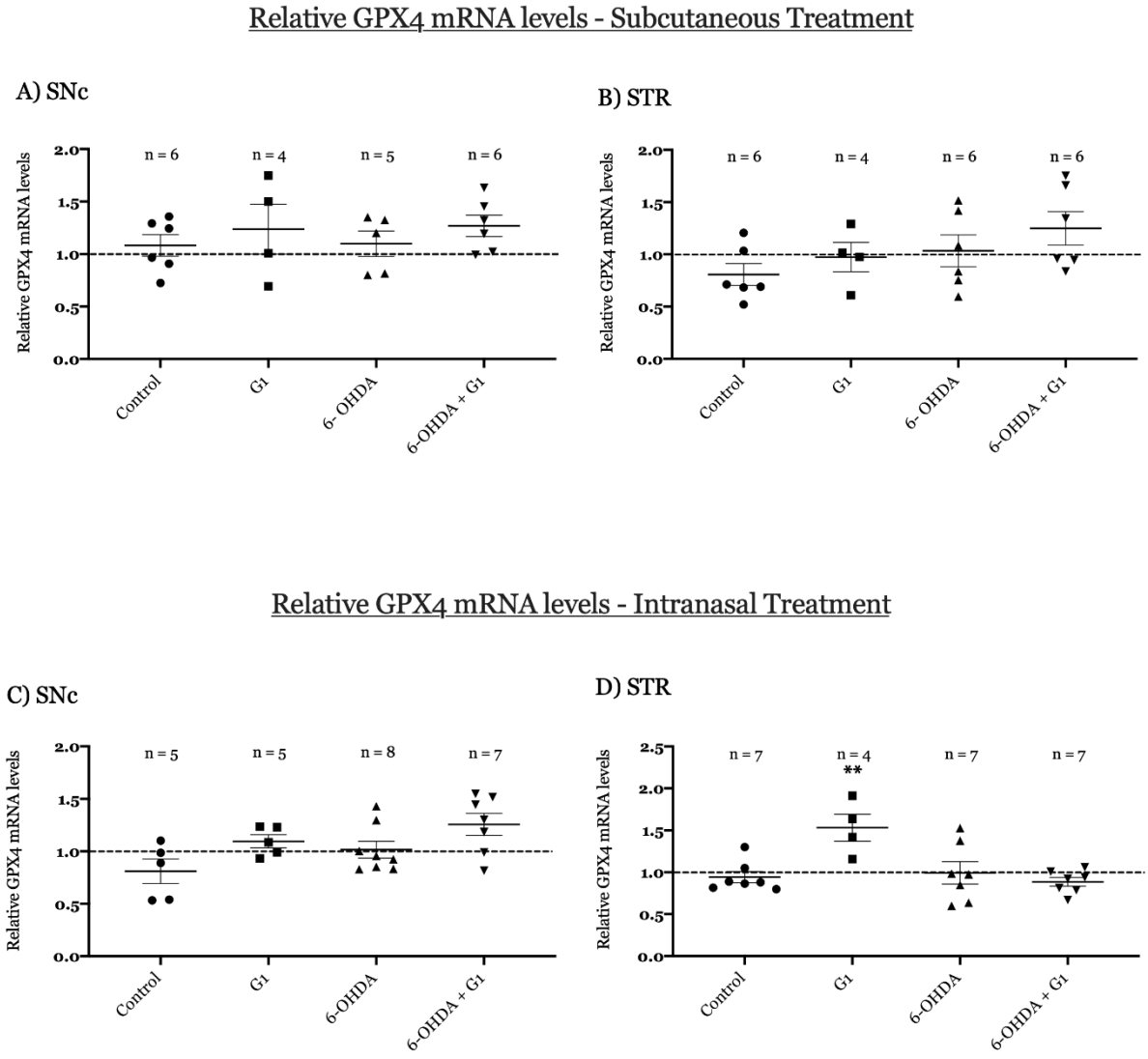

Figure 14. Effect of GPER activation on mRNA levels of the glutathione peroxidase 4 in animals subjected to Subcutaneous (A, B) and Intranasal (C, D) administration of G1. The animals were subjected to a stereotaxic injection of vehicle or 6-OHDA and were later treated with G1 or vehicle. At the end of the treatment, the animals were sacrificed and the regions corresponding to the substantia nigra (SNc) and striatum (STR) were collected. The values were normalized to the ipsilesional side of each animal (left). Data represents the mean ± standard error of the mean (SEM) of five to eight animals from different experimental conditions. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. (**p < 0.001 compared to Control).

GPX4 is a phospholipid hydroperoxidase that catalyzes the reduction of hydrogen peroxide, organic hydroperoxides, and lipid peroxides at the expense of reduced glutathione, protecting cells against oxidative stress.

Analysis of GPX4 mRNA levels in the SNc and STR were relative stable, with no major changes being induced by 6-OHDA injection or by the exposure to the GPER agonist, either subcutaneously or intranasally (Figure 14 A, B, C and D). The only exception was observed in STR tissue from animals subjected to intranasal administration of G1, in which a 62.42% increase of mRNA GPX4 levels was observed, when compared to control animals (Figure 14 D).

4.2.4. Relative NRF2 mRNA levels

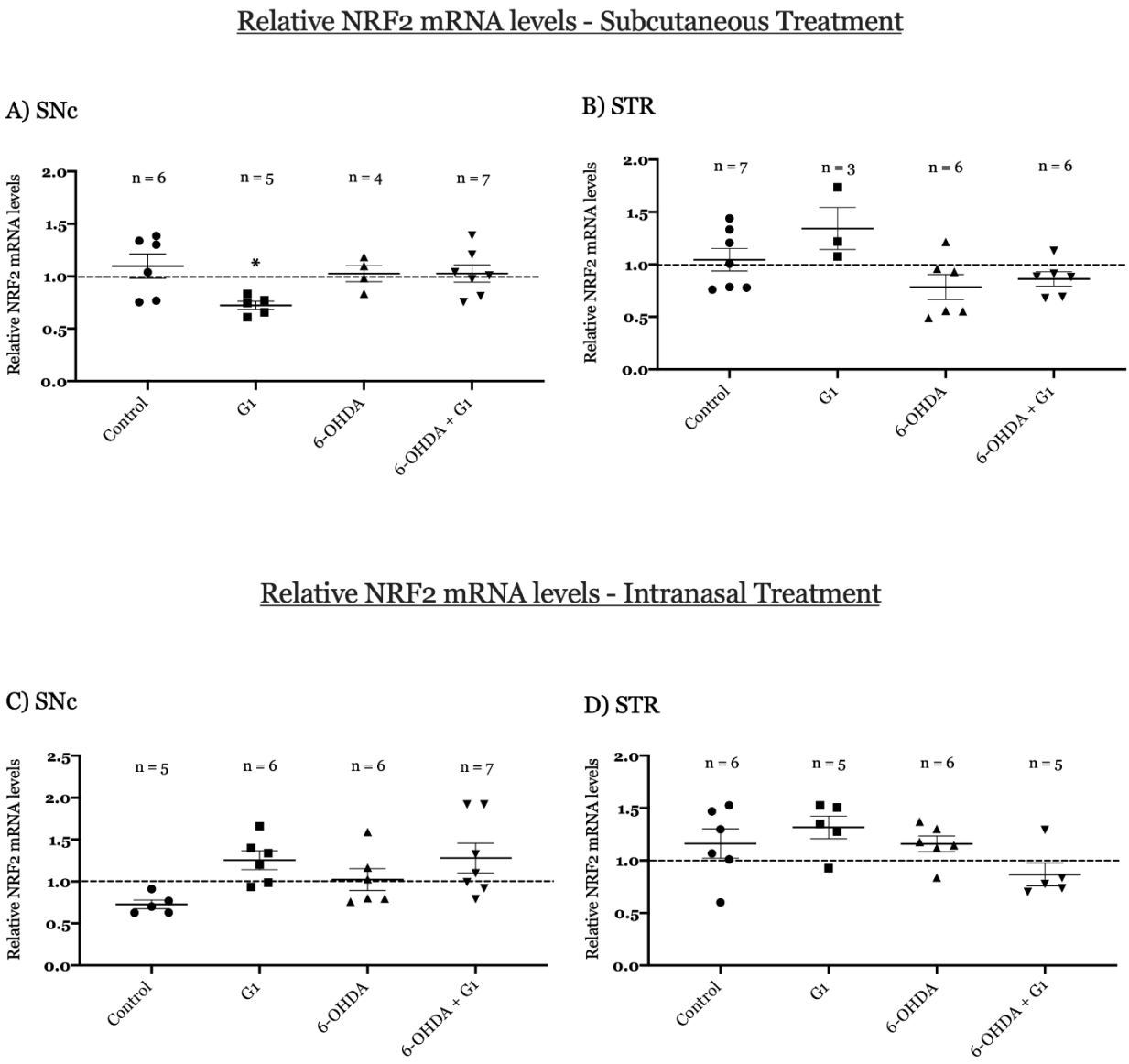

Figure 15. Effect of GPER activation on mRNA levels of the erythroid 2–related factor 2 in animals subjected to Subcutaneous (A, B) and Intranasal (C, D) administration of G1.
The animals were subjected to a stereotaxic injection of vehicle or 6-OHDA and were later treated with G1 or vehicle. At the end of the treatment, the animals were sacrificed and the regions corresponding to the substantia nigra and the striatum were collected. The values of the nigra were normalized to the left nigra (A, C) and the values of the striatum were normalized to the left striatum (B, D). Data represents the mean ± standard error of the mean (SEM) of five to seven animals from different experimental conditions. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. (*p < 0.05 compared to Control group).

Nrf2 is a main regulator of antioxidant defences. In both SNc and STR the mRNA levels of Nrf2 were similar, with the exception of the SNc of animals subjected to subcutaneous treatment with G1 in (Figure 15 A), which presented a 34.17% decrease when compared to the control group. Furthermore, the SNc of G1 group exhibited a 72.42% increase in the Nrf2 mRNA levels when compared with the control group (Figure 15 C).

4.3. Immunohistochemistry

In order to evaluate whether the selective activation of GPER with G1 has a neuroprotective effect on dopaminergic neurons, and if this effect is related to a decrease in oxidative stress, we performed an IHC assay against TH, a marker of dopaminergic neurons, and against 8-oxoG, a marker of oxidative damage to DNA.
Unfortunately, due to time constraints, it was not possible to quantify the results, and we only present a representative image of the staining obtained.

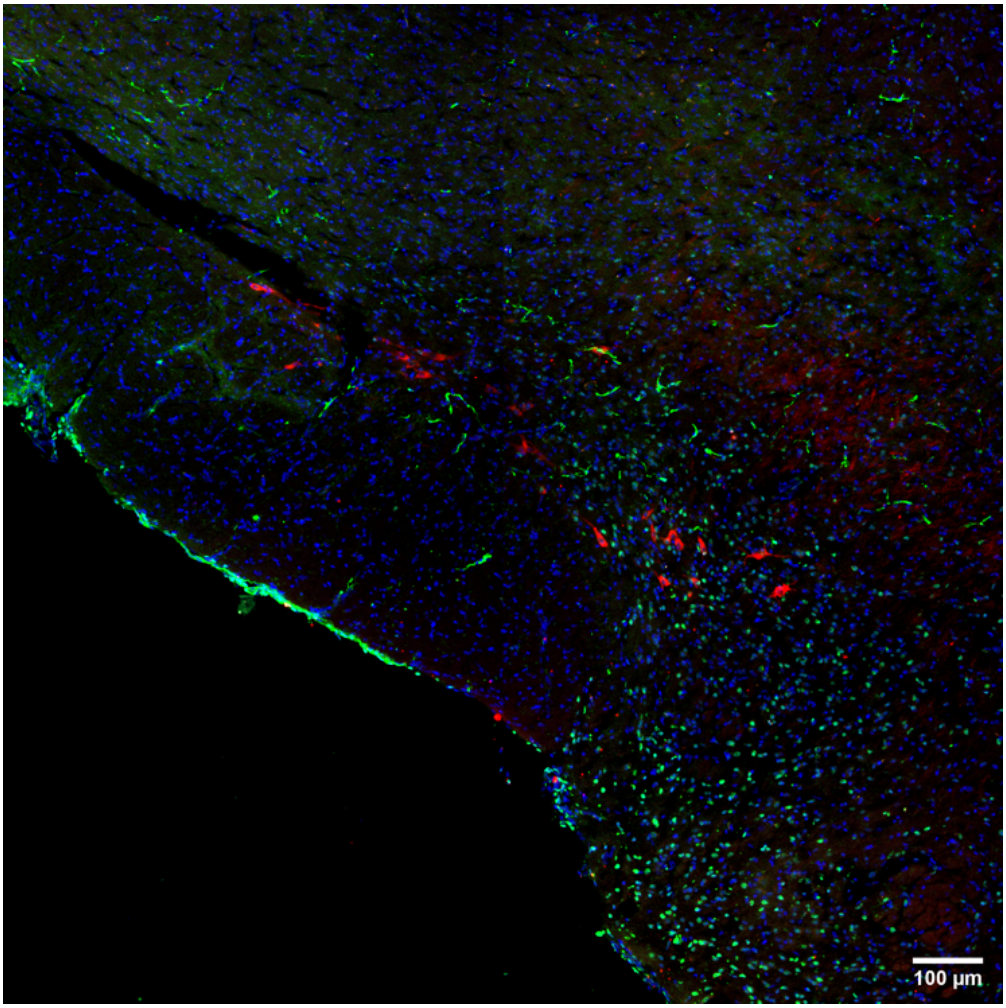

Figure 16. Representative image of the IHC using the TH marker to quantify dopaminergic neuronal loss, and 8-oxoG to quantify DNA oxidation. Fluorescent images from the sections were obtained with an LSM 710 confocal microscope (Zeiss) with 20x and 40x objectives. In the image we can see the nucleus stained with 33342 Hoechst (blue), TH in red and the 8-oxoG in green.

Chapter 5

Discussion

Discussion

Oxidative stress is considered a crucial factor in neurodegenerative diseases. In normal conditions, there is a balance between the production and the removal of reactive species. However, changes in this balance can lead to the accumulation of free radicals, promoting oxidative stress and, consequently, lipid peroxidation of cell membranes, alteration of structural and signaling proteins, leading to their aggregation, and oxidation of DNA/RNA that may lead to interruption of transcription or promotion of genetic mutations. In PD, increased oxidative stress was associated with the main characteristics of the disease: degeneration of SNc dopaminergic neurons, dopamine reduction in STR and formation of Lewy bodies.

In the current project, in order to induce parkinsonism, was used the intrastriatal injection of 6-OHDA, characterized by high levels of oxidative stress. Using this model, our main objective was to determine if the activation of GPER was able to modulate the damage caused by toxin-induced oxidative stress. To this purpose, we started by performing two types of administration of the agonist of this receptor: subcutaneous and intranasal.

As said before, changes in motor behavior are predominantly a consequence of the loss of dopaminergic neurons in SNc, so by inducing dopaminergic injury, we would expect to find changes in motor behavior. To assess behavioral changes, we performed the Grip test, Rotarod test and determined the total distance traveled in the Open field test. Contrary to what was expected[166–170] both the total distance travelled in the Open field test and the latency to fall in the Rotarod test were not affected by 6-OHDA injection. In comparation to the behavioral tests described above, the Grip test evaluates more detailed movements such as capacity to apply maximum isometric force of the hands and forearm muscles[171] to support the body weight. This test showed a decrease in latency to fall in 6-OHDA-injected mice. Taken together, the results do not show clearly that a significant dopaminergic injury was induced by the 6-OHDA injection. It would be important to confirm the extent of the induced injury by evaluating the number of dopaminergic neurons in the SNc and in the STR using immunohistochemistry against TH. Since our data does not allow us to conclude whether dopaminergic injury has occurred, it is not possible to determine if the activation of GPER receptor had any impact on it.

Since PD is characterized by the appearance of motor and non-motor symptoms, other types of tests were performed to assess non-motor changes. As previous studies shown an increase in anxious behavior in 6-OHDA-injected rodents, when subjected to the elevated plus maze,[172,173,174] we decided to study this behavior as well. Our results indicate a decrease in the number of elevations in the Z axis as well as in the distance traveled in the center of the arena for 6-OHDA-injected mice, which is in line with published studies.[175,176] This

means that the animals injected with the 6-OHDA presented more anxious behavior and, therefore, tend to stand near the periphery, having less interest in exploring the arena.
To our surprise, in contrast to the lack of a clear effect of 6-OHDA injection on motor parameters, 6-OHDA was effective and promoted more anxious behavior.
Next, we assessed whether 6-OHDA injection promoted changes in the oxidative defenses. Since increased oxidative stress was found in the brains of PD patients, antioxidant enzymes can be expected to be decreased and free radicals increased, representing the oxidative imbalance.[177] As PD is characterized by an accentuated decline of the antioxidant system[85,86] resulting in a severe worsening of ROS production and disease progression, it would be expected that the 6-OHDA-injected mice would present reduced mRNA levels of antioxidant enzymes.[93,95,178–181] To evaluate this, we used RT-PCR to assess the relative mRNA levels of enzymes crucial for antioxidant defense such as SOD1, CAT, Gpx4 and the transcriptional regulator Nrf2. Although not significantly, for SOD1 and CAT it was observed that 6-OHDA-injected mice presented decreased mRNA levels while Gpx4 and Nrf2 presented an increase. These changes can be justified by the way the antioxidant enzymes act. It has been described that, in some cases, when an injury occurs, the oxidative system acts by increasing its defenses as a form of neuroprotection, and only later, as time and injury increase, will the antioxidant enzymes decrease.[182,183] Nrf2 is a transcriptional factor responsible for the transcription of some antioxidant enzymes contributing crucially to antioxidant defense mechanism[89,90] and studies have shown that failure in its transcriptional activity makes dopaminergic neurons susceptible to oxidative stress damage.[93] Since an increase in Nrf2 mRNA levels was observed, this may indicate that a protection mechanism is being induced with the aim of increasing antioxidant defenses.
Studies have shown that the genetic elimination of GPX4 is lethal in the embryo[184,185], and the elimination of GPX4 in the brain results in extreme neurodegeneration, indicating that the role of GPX4 in the removal of lipid hydroperoxides is essential for cell viability. While there are studies showing that GPX4 mRNA levels are decreased after a dopaminergic insult [186], the increase observed in our results may indicate that a mechanism has occurred to protect the neurons from the oxidative stress induced by the toxin. This could also indicate that 6-OHDA-induced a mild lesion, that initiated neuroprotective mechanisms.

We also evaluated, by IHC, the staining for 8-oxoG and 4-hydroxynonenal (4-HNE), markers of nucleic acids and lipids oxidation, respectively.[177] These results are crucial to confirm that exposure to 6-OHDA indeed induced a significant increase in oxidative stress. Unfortunately, due to time constraints, it was not possible to complete this analysis.

Regarding the treatment with the GPER agonist (G1), in the behavior tests, the G1 group presented a decrease in the distance traveled in the center of the arena, suggesting that treatment with G1 may induce an anxious behavior. Since GPER is widely expressed and its activation can be involved in several processes such as cognition, memory consolidation[187] and spatial recognition memory[188] among others, G1 may be exerting its effects in other brain regions.

The changes in the mRNA levels of SOD1, CAT, Gpx4 and Nrf2 in animals subjected to subcutaneous and intranasal administration of G1 were analyzed in the SNc and STR. Although the 6-OHDA injection affects several structures, such as cortex and hippocampus[189], it is known that degeneration will be more evident at the injection site, in this case the STR. On the other hand, SNc is more susceptible to oxidative stress than STR, which may justify more pronounced changes in SNc than in the STR.[8,190]

It has been shown by our group and others that subcutaneous injection with G1 is effective in protecting dopaminergic neurons against toxins such as MPTP[152,191,192] and LPS[193] demonstrating an efficient G1 delivery to the brain. Moreover, although not published, a study from our group also showed an efficient G1 delivery to the brain by intranasal administration.[194] In the present study, the subcutaneous administration of G1 in 6-OHDA-injected mice induced an increase in the mRNA levels of SOD (figure 12 B), suggesting that G1 administered subcutaneously reached the nigrostriatal area and was able to modulate the expression of this enzyme.

As the subcutaneous injection is an invasive method, it would be important to use a less invasive method. Being a lipophilic substance[195,142], when subcutaneously injected, it will disperse throughout the body and may accumulate in various tissues. Since GPER is widely expressed in the body, G1 can trigger effects in other organs.[129] Besides, a greater dispersion would lead to a need of higher dose to promote brain effects.[196]

Intranasal administration overcomes some of the disadvantages of subcutaneous injection.[196,197] This route of administration has been used to direct drugs to the brain[198,199]. It is considered a less invasive technique and since the nasal mucosa is extremely irrigated and presents olfactory receptors that are connected to brain regions, it is considered a more direct and efficient delivery route to the brain. [196,200] However, as the nasal cavity is an highly irrigated area it also allows a fast distribution of lipophilic substances to the bloodstream, with subsequent distribution to all organs. It is also important to note that intranasal administration of compounds is challenging, in particular the control of the dose administered. The compound applied to the nostrils can easily be swallowed, come out instead of being absorbed or can be sneezed by the animals, thus altering the final dose applied.[200] The fact that G1-treated group presented less movements in the center of the

arena, when compared to the control group, demonstrates that the substance was able to reach the brain and alter mice behavior (figure 10 C and D).

Regarding the mRNA levels of the antioxidant enzymes, the fact that there was an increase in Gpx4 induced by G1 administration, when compared to the control, and no significant changes in the 6-OHDA + G1 group may indicate that in the presence of the toxin G1 was not capable of modulate the expression of this enzyme. However, although there are no effects on mRNA expression at the time point analyzed, there may be increases in the protein levels of these enzymes, or in its activity.

In conclusion, our results demonstrate that G1 had effects when injected both intranasally and subcutaneously. However, it is not possible to conclude whether the two G1 delivery approaches have an antioxidant effect in the presence of a dopaminergic insult. In this sense, further studies are needed to clarify whether the activation of GPER has an antioxidant effect, and whether this effect is related to its recognized neuroprotective effects.

Chapter 6

Future Perspectives

Future perspectives

In the present project, we used the 6-OHDA mice model of PD to evaluate the effect of GPER activation promoted by the administration of an agonist of this receptor by two routes, subcutaneous and intranasal. A batch of animals was used to assess the mRNA levels of antioxidant enzymes (SOD1, CAT, Gpx4) and the transcription factor Nrf2, as well as the motor and anxious behavior. A second batch of animals was used to evaluate the number of dopaminergic neurons, by IHC for TH, and the levels of 8-oxoG and 4-HNE, also by IHC. Unfortunately, due to the confinement caused by the COVID-19 pandemic, for the second batch of animals it was only possible to optimize the IHC assays, and it was not possible to analyze the results. Part of the proposed approaches are related to these tests that could not be concluded. Thus, to continue and complete the work presented in this thesis, we propose the implementation of the following approaches:

- To quantify the extent of the lesion between the different experimental groups, DA neurons in the SNc should be assessed by IHC against TH.

- To verify whether the 6-OHDA injection induced significative increases in oxidative stress, and if GPER activation can reverse this effect, it would be crucial to evaluate the levels of ROS in the SNc and in the STR as well as the direct consequences of oxidative stress, by measuring the levels of 8-oxoG and 4-HNE, indicators of nucleic acid and lipid oxidation respectively. It would also be important to complement our results with the determination of protein levels of SOD1, Gpx4, CAT and Nrf2 by Western Blot.

- In addition, it would be important to evaluate the distribution of G1 in the different organs and tissues of the animal when the compound is administered subcutaneously and intranasally. Preliminary results from our group showed that for the doses used in our studies the concentration of G1 present in the tissues of the animals is extremely low, with quantification requiring techniques such as HPLC coupled to mass spectrometry, to quantify the G1 accumulated. These data would allow to clarify how the route of administration will affect the levels of the compound reached in each region, and if this correlates with functional parameters or parameters regarding the antioxidant defenses.

Chapter 7

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