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Licenciado em Bioquímica

Klotho: relevance in the control of neuroinflammation in Parkinson's disease

Dissertação para obtenção do Grau de Mestre em

Bioquímica para a Saúde

Orientador: Prof. Doutora Carla Sofia Pais Fonseca, UBI

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Rui Gaspar

UNIVERSIDADE NOVA DE LISBOA

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Dedicatória

Aos meus pais, avós e Catarina dedico esta tese.

Agradecimentos

Primeiramente gostaria de agradecer à minha orientadora Professora Doutora Carla Fonseca e co-orientadora Graça Baltazar pela oportunidade e por me terem aceite para integrar o seu grupo de investigação. A sua orientação, recomendações e exigências foram essenciais para o desenvolvimento desta dissertação, tendo-me ajudado também a crescer tanto a nível pessoal como profissional.
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À minha família que, apesar de não puder estar sempre presente fisicamente, sempre demonstrou o seu apoio, me levou a querer ser melhor e a fazer melhor. Sem eles não seria quem sou, nem poderia estar onde estou. Um obrigado enorme por tudo.
Last but not the least, agradeço do fundo do coração à minha namorada Catarina, por ser o meu apoio ao longo destes anos, por vários desafios, sem a sua determinação, confiança e amor não seria possível chegar a este dia. Obrigado por me levantares a cabeça quando só queria desistir e por teres confiança em mim mesmo quando eu a perdia.

Resumo

O envelhecimento é o principal fator de risco para o desenvolvimento da doença de Parkinson (DP), que se caracteriza pela degeneração progressiva dos neurónios dopaminérgicos da *substantia nigra pars compacta* que projetam para o estriado, levando à depleção da dopamina estriatal e, consequentemente, às disfunções motoras tipicamente observadas. A patogénese da DP não é totalmente compreendida, mas a neuroinflamação mediada pela microglia parece contribuir para a degeneração dopaminérgica. A Klotho é uma proteína antienvelhecimento que prolonga o tempo de vida, protege os neurónios do hipocampo e aumenta a cognição. Ela possui propriedades anti-inflamatórias nos rins; porém não existem dados relativos a esse efeito no sistema nervoso central, mais propriamente na via nigrostriatal. Assim, obtivemos culturas primárias de microglia do mesencéfalo ventral e avaliámos o efeito da Klotho na reatividade microglial induzida pelo lipopolissacarídeo (LPS). Os resultados demonstraram que a Klotho inibe a expressão de iNOS, libertação de NO e atividade fagocítica induzidas pela exposição a LPS. Os recetores que poderão mediar este efeito anti-inflamatório não são conhecidos e foram alvo de estudo preliminar neste trabalho. Finalmente, no sentido de avaliar a relevância deste efeito anti-inflamatório da Klotho na neuroprotecção dopaminérgica exercida por esta proteína, procedemos à depleção da microglia numa cultura mista de neurónios-glia do mesencéfalo ventral. No entanto, tal depleção não foi eficiente não nos permitindo, por isso, inferir se o efeito anti-inflamatório da Klotho contribui para o seu papel neuroprotetor.

Os resultados apresentados neste trabalho mostram, pela primeira vez, que a Klotho é capaz de modular a reatividade microglial no mesencéfalo ventral podendo ser um potencial alvo terapêutico para o controlo da neuroinflamação associada à DP.

Palavras chave

Doença de Parkinson; Klotho; Neuroinflamação; Microglia

Abstract

Ageing is the primary risk factor for the development of Parkinson's disease (PD), which is characterised by the progressive degeneration of dopaminergic neurons in the *substantia nigra pars compacta* projecting to the striatum, leading to the depletion of striatal dopamine and, consequently, to the motor dysfunction typically observed. PD pathogenesis is not fully understood, but microglia-mediated neuroinflammation seems to contribute to dopaminergic neurodegeneration. Klotho is an anti-ageing protein that extends lifespan, protects hippocampal neurons and enhances cognition. It has anti-inflammatory properties in the kidney; however, there are no reports regarding this effect in the central nervous system, namely in the nigrostriatal pathway. Therefore, we obtained primary microglia cultures from the ventral midbrain and evaluated the effect of Klotho on LPS-induced microglial reactivity. The results showed that Klotho inhibits the LPS-induced iNOS expression, NO release and phagocytic activity. The receptors that may mediate Klotho's anti-inflammatory effect are unknown and were further explored in this work. Finally, in order to evaluate the contribution of Klotho's anti-inflammatory effect in its ability to protect dopaminergic neurons, we intended to eliminate microglia from a ventral midbrain neuron-glia mixed culture. However, microglia depletion was not effective which did not allow us to infer if Klotho's anti-inflammatory effect contributes for its neuroprotective role.

The results presented in this work show, for the first time, that Klotho is able to modulate microglial reactivity in the ventral midbrain, suggesting that it could be a potential therapeutic target for the control of neuroinflammation associated with PD.

Keywords

Parkinson's disease; Klotho; Neuroinflammation; Microglia

Communications

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Figure 4.7 Dopaminergic viability and microglial depletion with LME treatment. Mesencephalic neuron-glia mixed cultures were treated with 25, 50 or 75 mM LME for 1 h and allowed to rest with fresh NBM for 24 h. **A)** Representative images of the immunostaining for TH (DA neuron marker, green) and Iba1 (microglial marker, red), in which nuclei were stained with Hoechst 33342 (blue). **B)** Dopaminergic viability after LME treatment, as determined by the TH-immunopositive (TH^+) cell number, relative to the total number of cells ($Hoechst^+$), and expressed as a percentage of control (absence of LME). **C)** Microglial viability after LME treatment, as determined by the Iba1-immunopositive ($Iba\text{-}1^+$) cell number, relative to the total number of cells ($Hoechst^+$), and expressed as a percentage of control (absence of LME). Data are shown as mean ± S.E.M. of 3 independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA test followed by Bonferroni Multiple Comparison Test. (**$p < 0.01$ as compared to control). 39

List of abbreviations

BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
COMT	Catechol-ortho-methyl transferase
COX2	Cyclo-oxygenase 2
CSF	Cerebrospinal fluid
DA	Dopaminergic
DOPA	Dihydroxyphenylalanine
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FGFRs	Fibroblast growth factor receptors
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic factor
HUVECs	Human umbilical veins endothelial cells
IGF-1	Insulin-like growth factor-1
IL	Interleukine
iNOS	Inducible nitric oxide synthase
KL	Klotho
LBs	Lewy bodies
LPS	Lipopolysaccharide
MAO-B	Monoamine oxidase B
MEM	Minimal essential medium
MPP^+	1-methyl-4-phenylpiridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
m-KL	Transmembrane Klotho
NBM	Neurobasal medium
NDD	Neurodegenerative diseases
NED	N-1-naphthyl-ethylenediamine
NF-kB	Nuclear factor kappa B
NGF	Nerve growth factor
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with 0.1% Tween
PD	Parkinson’s disease
PDL	Poly-D-lysine
PFA	Paraformaldehyde
p-KL	Proteolyzed Klotho
Prx	Peroxiredoxin
ROS	Reactive oxygen species
s-KL	Secreted Klotho
SN	*Substantia nigra*

TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRPV5	Transient receptor potential cation channel subfamily V member 5
Trx	Thioredoxin
Trxrd-1	Thioredoxin reductase 1

Chapter 1

Introduction

1. Introduction

1.1. Parkinson's disease

Neurodegenerative diseases (NDD) are a heterogeneous group of chronic and progressive diseases characterized by a slow and gradual loss of neurons in discrete areas of the central nervous system (CNS), leading to disruption and progressive loss of specific functions associated with the affected CNS region, such as motor coordination, mobility, memory and cognition [1]. Due to genetic, environmental and endogenous factors, several processes, such as abnormal protein degradation and aggregation, oxidative stress, bioenergetic and mitochondrial dysfunctions, fragmentation of the neuronal Golgi apparatus, axonal transport disruption, alterations of molecular chaperones, dysfunction of neurotrophins, neuroinflammation, among others, can lead to neuronal loss in these diseases [2]. NDD such as Alzheimer's disease, Parkinson's disease (PD) and amyotrophic lateral sclerosis occur mainly in the later stages of life, with ageing being the major risk factor for the development of these diseases [3].

PD's prevalence has been increasing and is currently the second most prevalent neurodegenerative disease, right after Alzheimer's disease, being the most common movement disorder [4, 5]. In epidemiological terms, PD has a prevalence of approximately 0.2% of the general population, reaching about 1% in people over 60 years of age, and increasing to about 4% in the highest age groups [5]. Gender also seems to influence, with several studies reporting higher incidence in male subjects at a ratio of 2:1 [6], possibly due to the neuroprotective effects of estrogens [7].

James Parkinson was the first to clinically characterize the pathology by several motor changes, in the 19th century, being later deepened by Jean-Martin Charcot [8]. The main motor symptoms are tremor at rest, muscle stiffness, bradykinesia and postural instability, and usually occur asymmetrically [9]. Although PD is considered a movement disorder, and motor symptoms dominate the clinical picture, a variety of non-motor symptoms, which are undervalued and can anticipate motor symptoms, are also seen in patients, such as autonomic dysfunctions, cognitive and sensory deficits, psychiatric disorders, abnormal fatigue and sleep disturbances [10].

This pathology has an incidence of 10 to 20 cases per 100,000 people per year [5]. However, the existing treatment is merely symptomatic and ineffective at stopping the neurodegenerative process. Since the 1960's, the focus of the therapy at PD's early stages has been the use of drugs such as dopamine replacement or supplementation and direct stimulation of its receptors [11]. Even so, anticholinergics and enzyme inhibitors, such as dihydroxyphenylalanine (DOPA) decarboxylase, monoamine oxidase B (MAO-B) and catechol-ortho-methyl transferase (COMT), have proven to be an effective alternative in improving motor symptoms [7, 12]. Among all the pharmacological alternatives, L-3,4-dihydroxyphenylalanine (L-DOPA or levodopa) is the most frequently used. However, its prolonged use is often accompanied by motor complications, which include motor fluctuations and L-DOPA-induced dyskinesia, being required a posterior dosage adjustment or combination with other dopaminergic agonists, such as apomorphine, in order to control adverse symptoms [13]. However, although some PD's motor dysfunctions such as tremors and dyskinesia can be alleviated recurring to this therapy, postural instability and non-motor symptoms, namely neuropsychiatric disorders, are less sensitive and require other approaches. Recent research is focusing on addressing different non-pharmacological

strategies, recurring to deep brain stimulation, repetitive transcranial magnetic stimulation, gene therapy and cell replacement. Even more, neurotrophic and anti-inflammatory approaches have been suggested as neuroprotective therapies [14-16].

1.1.1. Neuropathology of Parkinson's disease

Neuropathologically, PD is characterized by the progressive loss of dopaminergic (DA) neurons of the *substantia nigra* (SN) *pars compacta* of the midbrain. These DA neurons project their axons mainly into the striatum (caudate-putamen), forming the nigrostriatal pathway. It is in the striatum that the depletion of dopamine occurs as a consequence of the degeneration of DA terminals, giving rise to the previously mentioned motor symptoms. First symptoms appear when the dopamine levels in the striatum are reduced by 80% or when 60% of the nigrostriatal DA neurons are lost [17]. Since the nigrostriatal pathway contains large amounts of neuromelanin, the accentuated loss of these neurons induces depigmentation of the SN *pars compacta*, which is characteristic of this disease [18] (Figure 1.1).

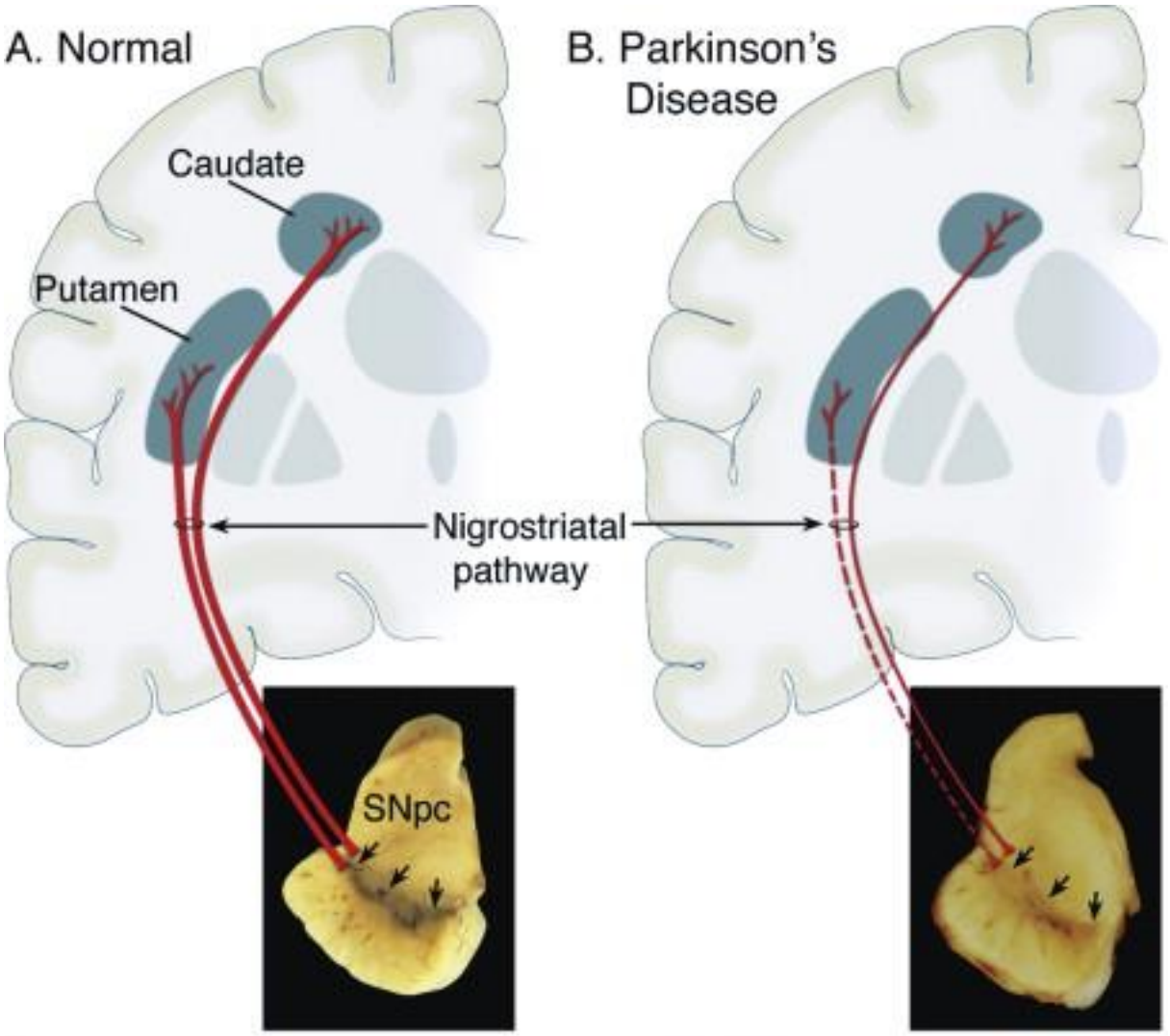


Figure 1.1 Neuropathological characteristics of PD. Schematic representation of normal nigrostriatal pathway (A) and characteristic of PD (B). The photographs show a pigmentation of the SN *pars compacta* (SNpc) in a healthy subject, resulting from the presence of neuromelanin in DA neurons, and a depigmentation in a PD patient. Adapted from Dauer and Przedborski [17].

In addition to DA neurons of the nigrostriatal pathway, other neurotransmitter systems, such as cholinergic, adrenergic and serotonergic, are also affected and cell loss is seen in other nuclei of the brainstem and cortex, being a major cause of non-motor symptoms [19].

This pathology is also characterized by the presence of neuronal eosinophilic cytoplasmic inclusions, called Lewy bodies (LBs), as consequence of an abnormal accumulation of a wide variety of proteins, mainly α-synuclein, a small protein widely expressed in the brain. However, the presence of these bodies alone does not present an explanation to the onset and progression of PD [20].

Pathologically, it has been suggested that both selective loss of DA neurons and accumulation of α-synuclein are influenced by several factors, including dysfunctions in the ubiquitin-proteasome system, mitochondrial dysfunctions, disorders of calcium homeostasis, neuroinflammation, and changes in protection mechanisms against oxidative stress and apoptosis [21-24].

1.1.2. Etiology of Parkinson's disease

Age represents the major predisposing factor for PD, possibly due to failures in normal biochemical cellular processes, through accumulation of mitochondrial DNA mutations and production of reactive oxygen species (ROS) which occur with ageing and render DA neurons more vulnerable to toxic aggressions [3]. Most cases of PD are idiopathic, yet there is evidence supporting that neurodegeneration is also related with a large variety of factors, including genetic, environmental and immunological factors [14].

Genetic factors appear to contribute in approximately 10% of PD cases. Mutations in the genes encoding α-synuclein, parkin, UCH-L1 (Ubiquitin C-terminal Hydrolase L1), PINK1 (PTEN-INduced putative Kinase 1) and LRRK2 (Leucine-Rich Repeat Kinase 2) have already been identified in familiar forms of PD where LBs are also identified, and may lead to malfunctions in the handling of proteins and consequently to degeneration [25]. The environmental factors that have been associated to PD are several, among them exposure to pesticides and use of β-blockers. Farming and well water exposure have also been associated, although that is most likely due to exposure to pesticides and other toxins [26]. On the other hand, there are several endogenous mechanisms that together can contribute to the pathogenesis, among them oxidative stress, mitochondrial dysfunctions, abnormal folding of proteins, cytoplasmic inclusions of proteins, excitotoxicity and loss of neurotrophic factors (glial cell line-derived neurotrophic factor (GDNF), neurturin, etc.) [27]. Recent studies point to a possible role of autoimmune mechanisms in the development of the disease. A significant increase in cytokine levels of innate immunity, including interleukin (IL)-1, IL-2, IL-6, and tumour necrosis factor (TNF) in SN and cerebrospinal fluid (CSF) have been observed in PD patients [14].

Despite all the processes that trigger PD, a prominent feature of several neurodegenerative diseases yet to be referred to, but rather important and increasingly pointed as a cause is neuroinflammation, in which chronic immune activation occurs, particularly microglia activation [28].

1.1.3. Neuroinflammation

Increasing evidence has emerged that progressive neurodegeneration that occurs over several years is also closely associated with chronic inflammation. The concept of inflammation, more particularly

neuroinflammation, has undergone a major transformation in recent years and is defined as the brain's response to injury, infection or disease. Neuroinflammatory mechanisms include the responses of microglia cells (reactive microgliosis), to a lesser degree by astrocytes (astrogliosis) and leukocytes. However, these changes are not PD specific and these processes may contribute to various neurodegenerative diseases [19].

1.1.3.1. Evidence on Parkinson's disease

Neuroinflammation is an obvious feature of this pathology, yet it has not been determined whether this neuroinflammation protects or promotes neurodegeneration. The first evidence of the involvement of neuroinflammation in PD appeared in 1988. By immunohistochemistry reactive microglia in the SN *pars compacta* was identified in *post-mortem* brains of PD patients [29]. Later, through the same approach, further studies also allowed to observe that this microglial activation was not limited to the SN, but was also extended to the putamen, hippocampus, transentorhinal cortex, cingulate cortex and temporal cortex [30]. Since then, several evidences support and increasingly confirm the idea of inflammatory responses mediated by microglia contributing significantly to the degeneration process. Changes in microglial activation that correspond to the loss of DA terminals in the nigrostriatal pathway at the onset of the disease [31] and increased concentrations of proinflammatory cytokines such as TNF-α, IL-1β, interferon γ (IFN-γ) in the nigrostriatal pathway, CSF and serum of PD patients [32], are examples of some findings. In fact, some studies have induced lower expression of these cytokines in the SN which resulted, as expected, in a delay of the progressive DA neurons degeneration and arise of motor symptoms [33].

Astrocytic reactivity is another known neuropathological characteristic of SN in PD. Although Mirza and his collaborators were unable to identify reactive astrocytes in the SN and in the striatum of *post-mortem* tissues of PD patients [34], further studies revealed an active astrogliosis that could maintain the degeneration of DA neurons, being reported that astrocytes even exacerbated microglia's effect regarding DA neurons degeneration [35]. Even so, in many cases of patients with PD it is observed a slight increase in the number of reactive astrocytes expressing the glial fibrillary acidic protein (GFAP), which additionally inversely correlates with DA neurons degeneration [36].

1.1.3.2. Role of glial cells: microglia and astrocytes

Microglia cells are CNS resident macrophages and one of the main types of cells that modulates inflammatory responses. The CNS environment is generally immunosuppressive, causing microglia to be maintained in a resting state, with a branched morphology, in relation to the other tissue macrophages. However, after their activation they acquire an ameboid morphology which facilitates migration to the lesion site, where it can perform important beneficial actions but, on the other hand, can also perform harmful actions [37].

Specifically in response to injury, activated microglia has the ability to surround damaged neurons and participate in their repair and development process by releasing neuroprotective factors, namely brain-derived neurotrophic factor (BDNF) and GDNF [38, 39]. In addition, these cells may contribute to the secretion of anti-inflammatory factors and also stimulate astrocytes to remove glutamate during an

inflammatory response. However, its persistent activation, such as in response to neuronal damage or environmental toxins, can amplify the progressive damage on surrounding neurons through the production of neurotoxic factors, such as glutamate, arachidonic acid metabolites, histamine and proinflammatory cytokines which induce the expression of inducible nitric oxide synthase (iNOS) or cyclooxygenase 2 (COX2), and ROS-producing enzymes [38, 40, 41].

Evidence indicates that microglia can acquire two distinct activation phenotypes, the M1 (proinflammatory) and M2 (anti-inflammatory) phenotypes. The M1 phenotype leads to the production of ROS, NO, chemokines, proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-1, and excitotoxins, in particular the previously mentioned glutamate, while the M2 phenotype leads to the release of anti-inflammatory cytokines, such as IL-4, IL -13, IL-10 and the transforming growth factor-β (TGF-β). Exposure to lipopolysaccharide (LPS)/IFN-γ has been reported to induce the M1 phenotype, whereas IL-4/IL-13 treatment induces the M2 phenotype [42]. Evidence from PD patients indicates that microglial proliferation occurs at the early stage of the disease and that it remains relatively static [43]. However, dopaminergic degeneration during disease progression has been associated with an increased production of proinflammatory cytokines, once again evidencing that microglia has the capacity to shift through distinct phenotypes throughout disease progression, being characterized by different amounts of cytokines secreted. However, to date, there have been few studies investigating therapeutic strategies aiming at changing microglial phenotype as a mean of reducing disease progression [44].

Astrocytes are present in all regions of the brain and play a crucial role in the homeostatic control of the extracellular environment in the CNS and, similarly to microglia, are also involved in neuroinflammatory processes. In response to pathological conditions, such as neuronal damage, toxic aggressions and ischemia, these cells become activated and have the capacity to proliferate and migrate to the lesion site [45]. This reactive astrogliosis has been verified in several animal models and is characterized by hypertrophic morphological alterations and by the overexpression of GFAP and the protein Connexin 43 [46].

Indeed, as with microglia, astrocytes also respond to inflammatory stimuli, including IL-1β and TNF-α, supporting the hypothesis that these cytokines may lead to astrocytic activation following CNS injury [39]. That is, the release of pro-inflammatory mediators by activated microglia itself, also contributes to reactive astrogliosis [35]. In addition, it is also reported that, at the same time, astrocytes secrete molecules with neurotoxic potential, such as NO, reactive sulphur species and various proinflammatory cytokines, both *in vitro* and *in vivo*, thus contributing to neuronal degeneration and neuroinflammation [35, 47]. On the other hand, a neuroprotective role is also attributed to astrocytes, since they additionally secrete anti-inflammatory cytokines and multiple neural protection agents, such as neurotrophic factors, including nerve growth factor (NGF), neurotrophin-3, GDNF, BDNF, among others, which are essential in neuronal protection and appear to be involved in the control of microglial activation [39, 48], thus guaranteeing the survival and correct functioning of neurons. In addition, astrocytes may further reduce oxidative stress and prevent NO production by a glutathione-dependent mechanism [39]. As so, an increase in reactive astrocytes in the SN of PD patients may indicate an attempt of neuroprotection in response to oxidative damage [49].

Therefore, uncontrolled neuroinflammation caused by the synergistic activation of microglia and astrocytes contributes in part to neurodegeneration of DA neurons in SN *pars compacta* (Figure 1.2). However, all these studies do not imply that neurodegeneration is merely a consequence of neuroinflammation. Other lines of evidence also suggest that neuronal degeneration may be the cause of inflammatory processes [37].

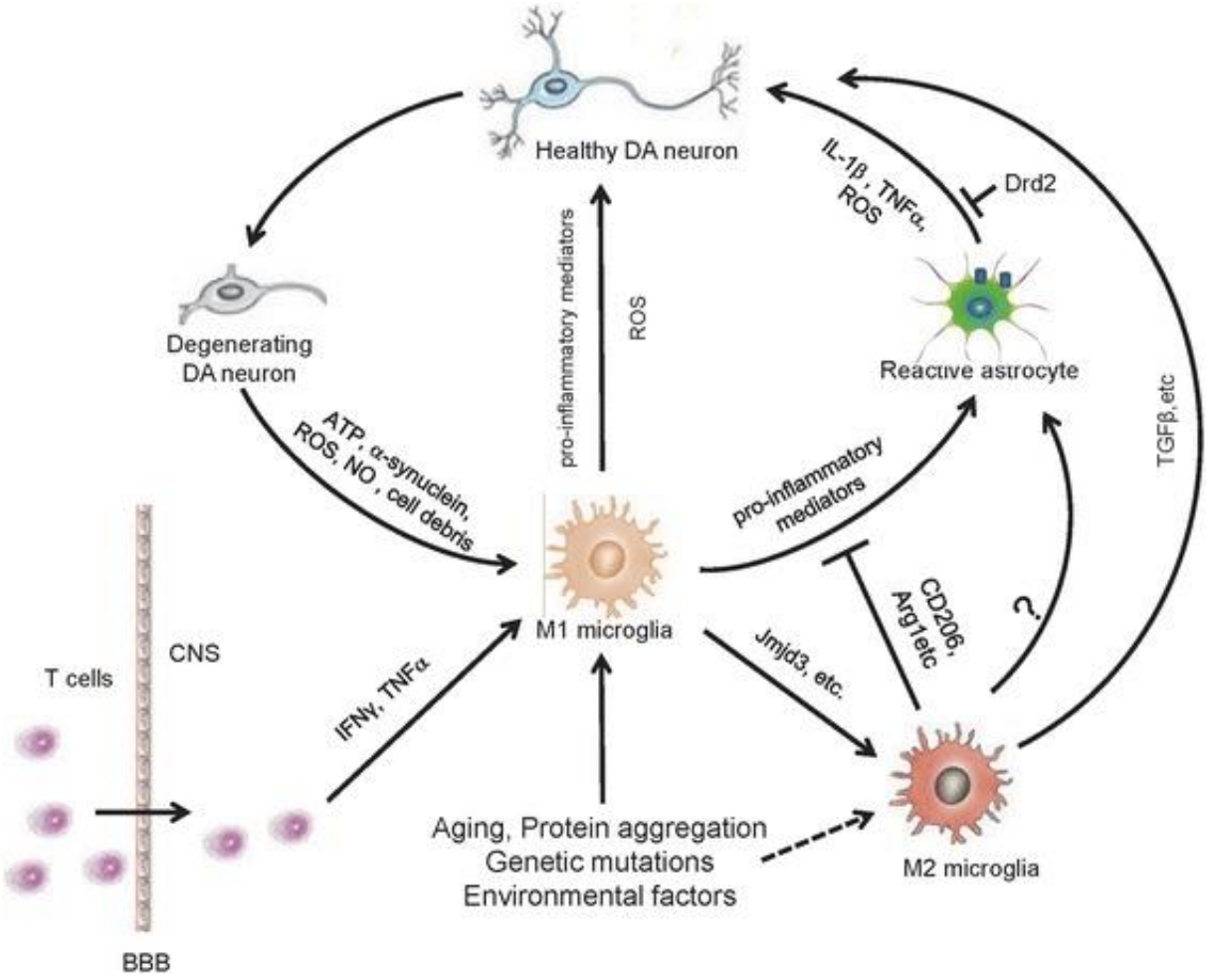


Figure 1.2 Representation of inflammatory mechanisms involved in PD pathogenesis. M1 phenotype is induced in PD under pathological conditions such as protein aggregation, gene mutations, environmental factors and cytokines release. M1 microglia activate astrocytes, by releasing pro-inflammatory mediators, leading to elevated production of proinflammatory factors, nitric oxide and superoxide radical, thus contributing to the degeneration of DA neurons. Damaged DA neurons can cause further activation of glia and enhance the inflammatory response. On the other hand, M2 phenotype can release anti-inflammatory factors, including TGF-β, preventing exacerbated reactivity and exerting a neuroprotective effect in PD [42].

1.1.3.3. Experimental model - LPS, a potent inducer of inflammation

LPS is a central component of the outer membrane of Gram-negative bacteria and frequently acts as an active endotoxin, being responsible for initiating a cascade of events during bacterial infections leading to the production of cytokines and other inflammatory mediators and increased levels of oxidative stress, which are also present in PD. It is reported that there are several LPS binding sites to the membrane, and some soluble proteins with the ability to bind to LPS have also been identified. The LPS-binding protein, the CD14 protein and the toll-like receptor (TLR) family appear to be among the

most important [50]. Microglial cells were recognized as the main brain cells responsive to LPS [35]. Therefore, upon LPS exposure, microglia become activated and release several cytokines (such as IL-1β, IL-6, TNF-α and nuclear factor kappa B (NF-kB)), increase the expression of iNOS (with consequent increase in NO production and release), prostaglandins and increase ROS release by NADPH oxidase, factors that together contribute to neuronal death [38, 50, 51].

Over the past years, studies have shown that LPS-induced inflammation can effectively replicate some of the features present in PD both *in vivo* and *in vitro*, including extensive microglial activation and progressive degeneration of the dopaminergic system [52]. On the other hand, *in vitro* studies from mouse mesencephalic cultures allowed researchers to conclude that there is a differential susceptibility of the brain regions to LPS. Although it appears to have an impact on midbrain neurons in general, there is a positive correlation between the region's microglial abundance and LPS-induced neurotoxicity [53]. As so, the LPS model is a useful tool in the study of mechanisms involved in microglial reactivity as well as in the study of mechanisms by which microglial reactivity can cause dopaminergic degeneration and ways to prevent it.

1.2. Klotho anti-ageing protein

Klotho (KL) is a protein whose gene was originally identified in 1997 when a spontaneous mutation in the promoter region in a strain of mice originated several ageing-like phenotypes in an autosomal recessive manner [54]. These phenotypes were not visible until 3-4 weeks of age, from where KL deficiency started to present symptoms such as growth retardation, atherosclerosis, cutaneous and gonadal atrophy, infertility, hypoglycaemia, osteoporosis, pulmonary emphysema, cognitive deficits, motor neurons degeneration, and premature death between 2 and 3 months of age [54]. On the other hand, it was seen in transgenic mice that when KL is overexpressed those animals exhibit an enhanced cognitive performance and oxidative stress resistance, as well as an increased lifespan between 20% to 30%. These findings suggested that reduction in KL levels is harmful to the whole organism and supported the idea that it plays an important role in the ageing process [55, 56].

The human KL gene is located on chromosome 13q12 and is highly conserved among species, having 98% homology between humans and mice. As so, human data are very consistent with those reported in experimental animals [57, 58]. In humans, KL serum levels decrease with age after 40 years [59-61], and this decrease can be observed in individuals with several age-related diseases such as cardiovascular and kidney disease [62, 63]. Thus, it has been recently suggested that serum levels of KL may serve as a biomarker of ageing [64]. However, only one case of human KL mutation has been described to date, but several single nucleotide polymorphisms have been detected in the gene of this protein and associated with human ageing [65]. Notably, some of these polymorphisms are associated with shorter lifetimes [66] and the increased risk of multiple age-related disorders such as coronary artery disease, osteoporosis and stroke [67].

1.2.1. Structure and expression of Klotho

In mice and humans, the KL gene is composed of five exons and encodes a 130 kDa type 1 transmembrane glycoprotein with 1014 and 1012 amino acids in length, respectively [54]. The

intracellular domain is very short with no known functional domains, and the extracellular domain is composed of two internal repeats (KL1 and KL2) with an amino acid sequence similar to the family of glycosidases I, which hydrolyse the β-glycosidic bonds in saccharides, glycoproteins and glycolipids. Three types of KL proteins have already been described, being transmembrane (m-KL), secreted (s-KL), both arising from alternative splicing, and proteolyzed/cleaved (p-KL) (Figure 1.3). These three proteins have already been detected in both humans and mice [57, 68, 69].

The linker region between the two replicates of the extracellular domain contains four basic amino acids (Lys-Lys-Arg-Lys), which represent a suitable site for proteolytic cleavage. As so, the extracellular domain of m-KL can be cleaved by membrane proteases, such as ADAM10, ADAM17 (also called TACE - tumor necrosis factor-α-converting enzyme) and BACE1, and be released into the extracellular space, giving rise to a 130 kDa or 70 kDa p-KL, if cleaved in the transmembrane segment or in the linker region, respectively [68] (Figure 1.3). In addition, p-KL has already been detected in the systemic circulation, urine and CSF, and can function as an autocrine and paracrine hormone in multiple tissues and organs [70]. Even more, it was also found that the KL gene encodes two other proteins, β-Klotho and γ-Klotho, which share homology with KL, both with a single transmembrane segment [67].

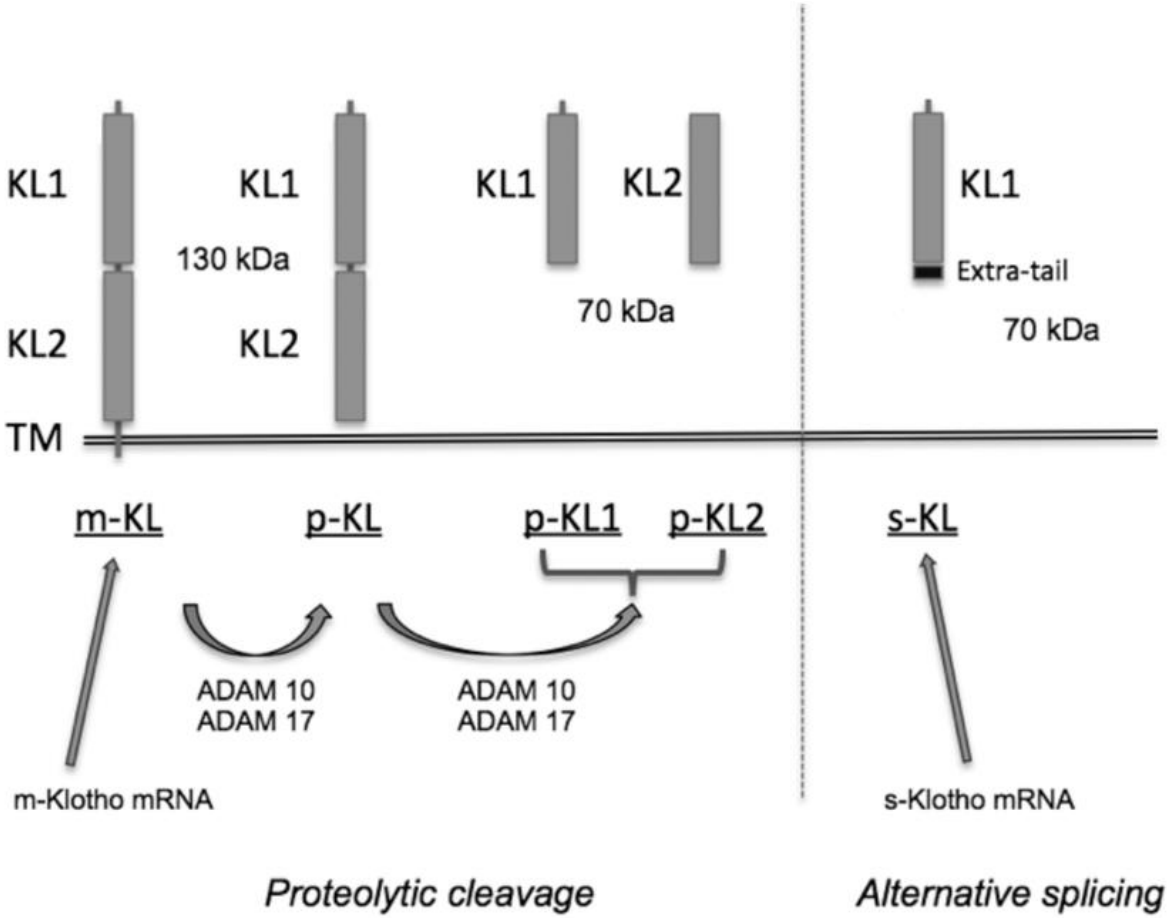


Figure 1.3 Schematic representation of Klotho isoforms. By alternative splicing two transcripts can occur. One of the transcripts encodes de transmembrane form (m-KL), which contains both KL1 and KL2 domains, while the second transcript encodes the secreted form (s-KL), which only contains the KL1 domain. The m-KL isoform may be cleaved by ADAM10 and ADAM17 α-secretases to produce the proteolyzed form (p-KL) that may be composed of both KL1 and KL2 or one single domain (either KL1 or KL2). Adapted from Massó et al. [68].

Currently, KL is known to be expressed in various tissues and cell types in mice, rats and humans, being predominantly expressed in the kidneys and brain [54, 71]. In addition, lower expression is detected in the pituitary gland, parathyroid gland, pancreas, ovary, testis, placenta, skeletal muscle, bladder, colon and breast epithelial cells [72]. Recently, this protein was also found to be locally expressed in the adventitial area of the aorta [73]. Particularly in the brain, in 2013, a study of the localization of KL mRNA in rat brains demonstrated that the choroid plexus is where it has greater expression [74]. Still, KL mRNA was also detected in other regions of the brain, such as the midbrain (with higher levels in the SN) and the striatum [74] (Figure 1.4). In addition, at the cellular level, it is in neurons, especially in the hippocampus and pituitary, and in Purkinje cells of the cerebellum that there is a greater expression of KL [54, 75, 76], while microglia and astrocytes do not express this protein [74].

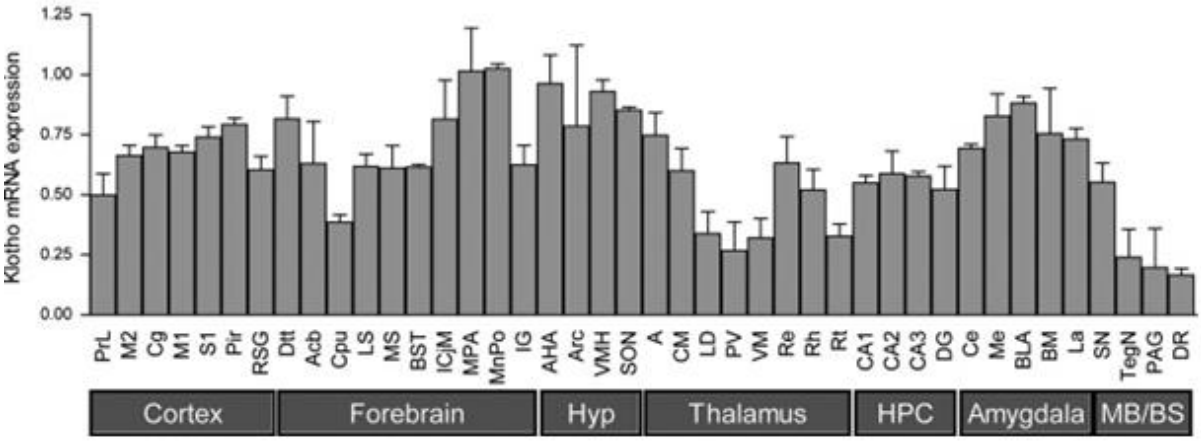


Figure 1.4 Klotho mRNA distribution by *in situ* hybridization in the adult rat brain. KL mRNA signal was quantified in several brain regions of adult rats (n=3–4). KL mRNA was widely expressed throughout the brain, including several cortical areas, forebrain structures (including the caudate-putamen (Cpu)), hypothalamus (Hyp), thalamus, hippocampus (HPC), amygdala, and midbrain/brain stem (MB/BS, including the SN). Adapted from Clinton et al. [74].

According to the analysis of several tissues, the main gene transcript of this protein is s-KL [57]. Recently, a study has shown for the first time that the soluble domain released by cleavage generates a stable protein and, in contrast to m-KL, is ten times more expressed in the brain than in the kidney [74], which suggests that these two Klotho forms may have different functions. In addition, a strong correlation between the levels of isoform expression and the health status of the animals under study was also observed [68].

As so, although the KL gene is only expressed in some tissues, it has been observed that the deficit of this protein leads to ageing phenotypes in almost all tissues and organs, confirming that it functions as a hormone and acts on tissues other than those where it is expressed. However, molecular mechanisms and their exact biological functions are not yet fully understood.

1.2.2. Functions of Klotho

The three possible forms of this protein can perform different functions. On one hand, m-KL forms complexes with various fibroblast growth factor (FGF) receptors (FGFRs) in humans, namely FGFR1c, FGFR3c and FGFR4, thereby selectively increasing affinity for the factor FGF23, a hormone that inhibits

phosphate reabsorption and vitamin D biosynthesis in the kidney, negatively regulating calcium homeostasis [69, 77]. Thus, the deficit in KL leads to increased production of 1,25(OH)2D3, an active metabolite of vitamin D that plays an important role in calcium metabolism [54] and increased oxidative stress [55]. Overproduction of 1,25(OH)2D3 may be the main responsible for development of ageing phenotypes such as hyperphosphatemia and hypercalcaemia, similar in KL-deficient mice [54]. Thus, without KL, the function of the FGF23 hormone is literally nullified.

On the other hand, p-KL functions as a hormone, independent of FGFRs, with different activities in several target cells. However, it has been reported that p-KL can still interact with FGFRs being an impactful factor in FGF23 signalling [78]. Firstly, this isoform negatively regulates the signalling pathway of insulin-like growth factor-1 (IGF-1)/insulin [55]. One study revealed that moderate inhibition of the IGF-1/insulin signalling pathway is one of the most evolutionarily conserved mechanisms to suppress ageing and extend life span [79]. It has been suggested that KL can bind with high affinity to receptors expressed on the surface of cells, although yet unidentified, and consequently activate the IGF-1/insulin signalling pathway. Although the exact mechanism has not yet been determined, it is thought that it may involve resistance to oxidative stress levels in mammals by reducing ROS production [80]. On the other hand, p-KL also has a role in ion homeostasis, through the regulation of ion channels and transporters, namely the transient receptor potential cation channel subfamily V member 5 (TRPV5), emphasizing once again with an antioxidant role. In addition, TRPV5 plays a role in the maintenance of calcium homeostasis through its reabsorption of intracellular space. In particular, β-glucuronidase activity of KL plays an important role in the activation of this ion channel [81]. Even more, this protein also has the ability to regulate NO production since studies have shown that levels of NO metabolites in urine, such as NO_2^- and NO_3^-, were significantly reduced in KL-deficient mice. However, the mechanism of KL-mediated NO synthesis is still unknown [82].

Concerning the s-KL, it has been shown that in some tissues, such as the kidneys and the choroid plexus, it interacts with the Na^+-K^+ ATPase pump in intracellular organelles, but not in the plasma membrane, and stimulates its expression and activity, thus facilitating Ca^{2+} transport [83]. It is also reported that the s-KL isoform can interact with the retinoic acid-inducible gene-I (RIG-I) and inhibit the expression of inflammatory cytokines such as IL-6 and IL-8 in human umbilical vein endothelial cells (HUVECs) and fibroblasts. In addition to these, the expression of other cytokines is also inhibited by KL, such as TNF-β and IFN-γ [67, 84]. This data supports the idea that Klotho may function as an intracellular anti-inflammatory and anti-ageing factor.

Finally, recent studies have shown that KL may influence other signalling pathways, such as the p21/p53 signalling pathway, regulating several genes involved in the cell cycle, the cyclic adenosine monophosphate (cAMP) pathway and the protein kinase C (PKC) and Wnt pathway in HUVECs and fibroblasts [69].

The biological function of KL protein and the mechanism by which its absence contributes to neurodegenerative diseases are still unknown. However, of all the above-mentioned functions, it is important to note that the anti-ageing properties attributed to KL seem to be closely associated with increased resistance to oxidative stress and its anti-inflammatory role, although the anti-inflammatory effect is widely unexplored in the CNS, namely in the ventral midbrain.

1.2.3. Evidence of Klotho's action on neuroprotection

It is important to note that neuronal KL may play an important role in ageing phenotypes and protect against the development of age-related neurodegenerative diseases. However, contrary to the kidney, where the function of KL protein has been further studied, there is currently no consensus on its role in the brain, especially in the susceptibility of the dopaminergic system in PD. Even more, the majority of the evidence correlating KL with the CNS comes from studies in knockout animals, deficient or with overexpression of KL.

When performing knockout mouse assays for KL, it was reported the presence of neuronal degeneration in some regions of the CNS, essentially in the hippocampus, and fewer Purkinje cells in the cerebellum [54, 85]. Reduced levels of anti-apoptotic proteins, glial filaments as well as GFAP in astrocytes were also some of the observations, which are curiously similar in elderly animals and humans [86]. Furthermore, other studies in the brains of these knockout mice have further demonstrated that in addition to neurodegeneration, there is a reduction in synaptic protein expression, axonal transport disorders [76, 87] and increased markers of apoptosis and oxidative stress [88].

More recently, TNF has been shown to negatively regulate KL expression via NF-κB and interestingly, studies have shown that, like other inflammatory diseases, this regulation remains in patients with chronic inflammation of the hippocampus [89]. Analysis of the antioxidant role of KL, when added to cultures of hippocampal neurons, revealed that KL significantly increases the expression of the thioredoxin/peroxiredoxin (Trx/Prx) system through the induction of peroxiredoxins (Prx-2 and Prx-3) and of thioredoxin reductase 1 (Trxrd-1), antioxidant enzymes that together reduce ROS, and leads to protection of hippocampal neurons from glutamate-induced death. Thus, Prx-2 may be a key modulator of the neuroprotective antioxidant role of KL [90].

Other behavioural studies performed on knockout mice reported memory deficits, probably due to an increase in brain oxidative stress and changes in cholinergic function [88]. Using Alzheimer's disease models, it was confirmed that in the affected areas the expression of KL decreased significantly compared to healthy animals of the same litter. In addition, the practice of moderate continuous exercise in adulthood prevented the decrease of both forms of KL, m-KL and s-KL [68].

It is also reported that, specifically in the white matter of monkey, rat and mouse brains, a decrease in KL with ageing is observed, probably due to the loss of myelin that occurs with age [91, 92]. In addition, it has been shown in cell cultures that KL enhances the maturation and differentiation of oligodendrocytes and myelination, while KL knockout mice brains express lower levels of mRNA and myelin proteins [76, 93]. Interestingly, studies tried limiting KL expression to the brain and testis and reported phenotype improvements compared to the knockouts, thus suggesting the importance of KL expression in the brain for the CNS ageing [54].

A study published in 2011 showed that KL mutant mice had a decrease in the number of DA neurons in SN and levels of dopamine in the striatum, which is possibly related to KL-mediated regulation of vitamin D, where an exposure to vitamin D would be the cause of the observed phenotype. In addition, they found that dopamine levels in the striatum significantly decreased with ageing in these animals [85]. Recently, it was shown that overexpression of KL, *in vivo*, significantly protected DA neurons against

oxidative damage induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in part through the modulation of p38 MAPK activation, an important pathway in the regulation of oxidative stress [94].
Given this, and considering all observations, it is found that the absence of KL has a great impact on the morphology and functioning of some cells, such as neurons and oligodendrocytes, and consequently health.

1.2.4. Evidence of Klotho in inflammation

Inflammation is one key factor associated with ageing and, as so, KL started being hypothesized as an anti-inflammatory protein as well. The first reports of KL having anti-inflammatory properties came from the kidney, one of the organs were KL is mainly expressed [54], where it was reported to negatively regulate the production of NF-kB–linked inflammatory proteins [95], which has also been recently reported for cardiomyocytes [96]. In the last years a limited number of articles have been suggesting KL as possible therapeutic target for neuroinflammation with a recent work reporting that KL could not only be involved in the modulation of microglial reactivity in the hippocampus, but also impacting the peripheral macrophages infiltration in the choroid plexus [97]. Still, there is an enormous lack of information regarding KL's anti-inflammatory properties in the CNS and, specifically, in the nigrostriatal pathway, even more if we take into account that microglia are regionally distinct.

Chapter 2

Objectives

2. Objectives

Reports have been describing KL's neuroprotective role as well as anti-inflammatory properties, with KL-deficient mice revealing greater DA degeneration while protecting DA neurons against oxidative damage when overexpressed. These results suggest that KL may play an important role in protection of DA neurons by modulating the inflammatory response and release of NO.

As so, this work's main objective is to evaluate if exogenously administered KL is able to modulate microglial reactivity *in vitro*, in primary cultures obtained from the ventral midbrain, and may be suggested as a new therapeutic target for PD. For that, the present work is guided by three specific objectives:

1) Evaluate if exogenously administered KL is able to modulate ventral midbrain microglial reactivity;
2) Explore possible receptors mediating KL's ability to modulate microglial reactivity;
3) Determine if the anti-inflammatory effect exerted by KL is relevant for its neuroprotective effect towards DA neurons.

Chapter 3

Materials and Methods

3. Materials and Methods

3.1. Animals for cell culture preparation

All animals used in the preparation of the cell cultures were treated accordingly to the protocols approved and ethical requirements for animal research and the European convention for the protection of vertebrate animals for experimental or other scientific purposes (Directive 2010/63/EU).

3.2. Primary microglia cultures from ventral midbrain

For each cell culture preparation postnatal day 2 to 4 Wistar rat pups were used. Inside a laminar flow chamber, to provide a sterile environment, the animals were sacrificed and the region corresponding to the ventral midbrain, comprised by the *substantia nigra* (SN) and ventral tegmental area (VTA) as seen in Figure 3.1, was dissected. Meninges were carefully removed, and the tissue was then minced with the scalpel and transferred to cold Phosphate Buffered Saline (PBS; 140 mM NaCl, 81 mM Na_2HPO_4, pH 7.4). Afterwards, the tissue was enzymatically digested in a papain solution (4 mg/mL papain, H&B solution (116 mM NaCl, 5.4 mM KCl, 26 mM $NaHCO_3$, 12 mM $NaH_2PO_4.H_2O$, 1 mM $MgSO_4.7H_2O$, 0.5 mM EDTA, 25 mM glucose, pH 7.3) and 0,5% phenol red in cysteine water (1.9 mM $CaCl_2$, 1.3 mM cysteine)) at 37 ºC for 4 minutes, shaken each minute, and then mechanically dissociated by sequentially passing it through micropipette (P1000) sterile tips with holes sized of 20G, 23G and 25G. To ensure no cell aggregates were left, the cell suspension was passed through a 70 µm mesh, and then centrifuged for 3 minutes at 405 xg (3K18C Bioblock Scientific; Sigma Laboratory Centrifuges). Supernatant was discarded and the remaining pellet was resuspended in M10C-G medium (2.2 g/L $NaHCO_3$, 0.75% glucose, 0.12% antibiotics (penicillin and streptomycin; Sigma) 0.02% insulin (Sigma) and 10% Fetal Bovine Serum (FBS; Biochrom AG) in Minimal Essential Medium (MEM). For cell counting the cell suspension was diluted (1:1) in a trypan blue solution (trypan blue 0.4% in NaCl 0.81% and K_2HPO_4 0.06%) and viable cells were counted in a Neubauer chamber. For immunocytochemistry and phagocytosis assay, cells were plated in 24 multiwell plates containing 13 mm or 10 mm coverslips previously coated with poly-D-lysine (PDL, 0.1 mg/mL; Sigma), at a density of 0.069×10^6 cells/cm^2, and maintained in culture at 37 ºC in a 5% CO_2 and 95% air atmosphere. For ROS measurements, cells were plated in 48 multiwell plates, previously coated with PDL 0.1 mg/mL (Sigma), at a density of 0.069×10^6 cells/cm^2. After 10 to 12 days in culture, when confluence was achieved, astrocytes were removed by mild trypsinization for 20 to 30 minutes, approximately, in a trypsin solution (0.125 g/L trypsin and 0.05 g/L EDTA in MEM). After this incubation period, detached astrocytes were discarded. The adherent microglia were kept in M10C-G medium at 37ºC in a 5% CO_2 and 95% air atmosphere for 5 days, to allow microglia to reach a resting state.

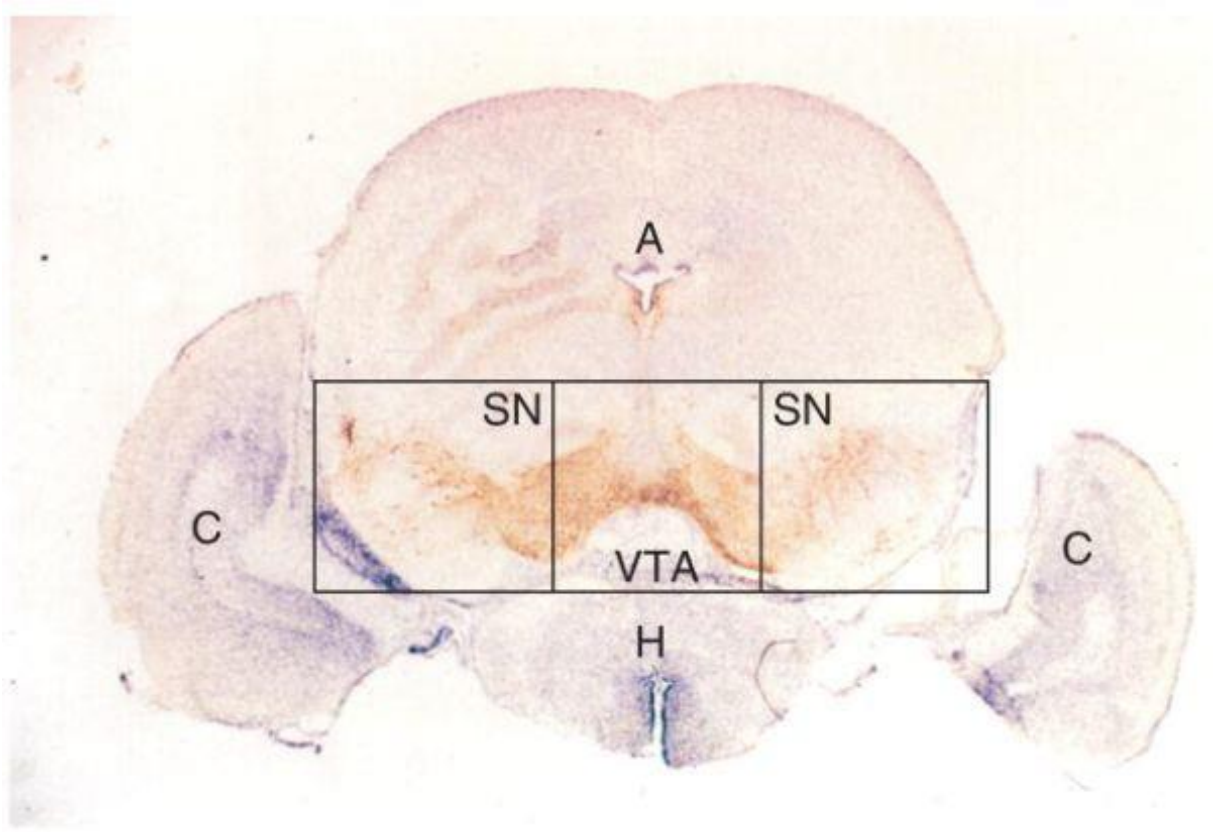


Figure 3.1 *Coronal section of postnatal day 1 mouse brain stained for tyrosine hydroxylase using horseradish peroxidase and diaminobenzidine. Black lines indicate the incisions made during the dissection. (A, aqueduct; C, cortex; H, hypothalamus; SN,* substantia nigra*; VTA, ventral tegmental area). [98]*

3.3. Primary neuron-glia cultures from ventral mesencephalon

For each cell culture preparation female Wistar rats with 15 or 16 gestation days were used. After anaesthesia with isoflurane the abdominal cavity was open, and embryos were removed. Afterwards, females were sacrificed via cervical dislocation. Uterine sacs were placed in cold sterile PBS.

Inside a laminar flow chamber, for a sterile environment, the amniotic membranes were removed and the region corresponding to the ventral mesencephalon was dissected, as represented in Figure 3.2. Meninges were carefully removed, and the resulting tissue was transferred to cold PBS and mechanically dissociated as described in section 3.2. The dissociated tissue was then centrifuged for 3 minutes at 405 xg (3K18C Bioblock Scientific; Sigma Laboratory Centrifuges). The supernatant was discarded, and the remaining pellet was resuspended in neurobasal medium (NBM; Gibco) supplemented with 2% B27 (Invitrogen), 0.05 µg/mL L-Glutamine (Sigma), 120 µg/mL gentamicin (Sigma), 25 µM L-Glutamic acid (Sigma) and 10% heat-inactivated FBS (Biochrom AG). For cell counting the cell suspension was diluted (1:1) in the trypan blue solution and viable cells were counted in a Neubauer chamber. Lastly, cells were plated at a density of 0.207 x 10^6 cell/cm^2 in 24 multiwell plates containing 13 mm or 10 mm coverslips previously coated with PDL 0.1 mg/mL (Sigma) and maintained at 37 ºC in a 5% CO_2 and 95% air atmosphere for 4 days.

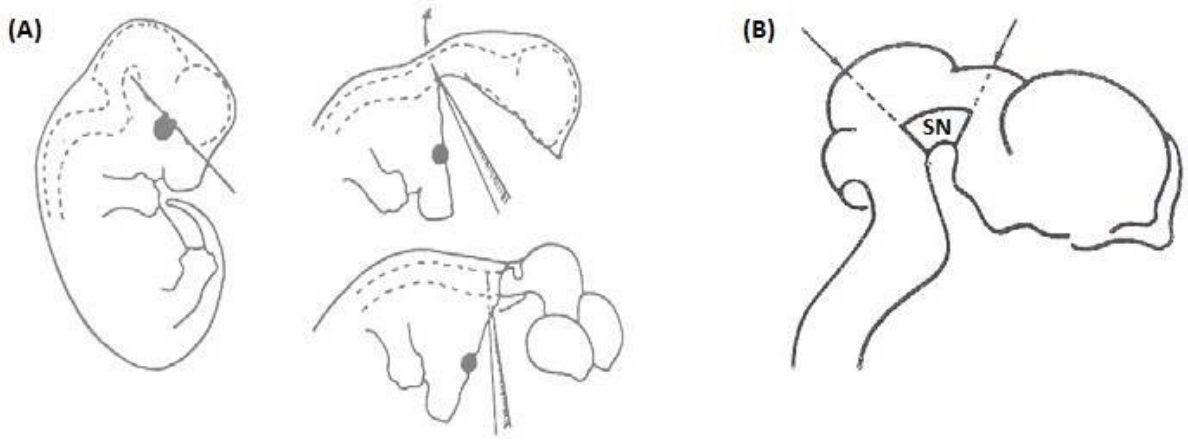


Figure 3.2 Ventral mesencephalon dissection from rat embryos with 15-16 days of gestation. (A) Representation of the incisions for rat embryo encephalon exposure. (B) *Substantia nigra* (SN) dissection from ventral mesencephalon. Adapted from Dunnet and Björklund [99].

3.4. Microglia cell culture treatments

Twenty-four hours prior to the experiments, primary microglia cell cultures had their culture medium changed to fresh M10C-G. Cells were then treated with 0.4 µg/mL of recombinant mouse KL (R&D Systems, ref.1819-KL-050) and 0.1 µg/mL or 1 µg/mL of LPS. Cells were exposed to LPS for 24 h to assess iNOS expression, NO production and phagocytic activity or 12 h to measure ROS production. KL was applied 24 h prior to LPS for iNOS expression, NO production, phagocytic activity and ROS measurement, or 1 h prior to LPS for iNOS expression, NO production and ROS measurement, or 1 h after LPS for iNOS expression, as indicated in figure legends.

To assess possible receptors that may mediate KL's anti-inflammatory effect three specific inhibitors, namely PD166866, Dovitinib and Roblitinib (MedChemExpress), of the receptors FGFR1, FGFR3 and FGFR4, respectively, were applied to microglia cell cultures at a concentration of 0.5 µM for PD166866 and 1 µM for Dovitinib and Roblitinib. One hour later, KL 0.4 µg/mL was added and 24 h later cells were exposed to LPS 0.1 µg/mL for an additional period of 24 h.

3.5. Microglia depletion in neuron-glia cultures

At the fourth day *in vitro*, primary neuron-glia cultures were treated with different concentrations (25, 50 or 75 mM) of L-leucine methyl esther (LME; Sigma) for 1h, after which cells were washed once with warm PBS to remove dead microglia cells. The remaining cells were then left in cultures for 24 h with an appropriate volume of NBM.

3.6. Immunocytochemistry

For the immunocytochemistry cells attached to coverslips were fixed with paraformaldehyde (PFA) 4% for 10 minutes. Afterwards, cells were permeabilized with Triton X-100 1% in PBS for 5 minutes at room temperature. A blockage for nonspecific interactions was performed with 20% FBS in PBS containing 0.1% Tween (PBS-T) for 1 hour at room temperature. After blockage, the cells were washed with PBS-T and incubated with the primary antibody (iNOS, mouse, 1:500, BD Biosciences;

Iba-1, rabbit, 1:2000, WAKO; TH, mouse, 1:1000, BD Biosciences) diluted in PBS-T containing 1% FBS, for either 1 hour at room temperature or overnight at 4 ºC, in accordance to manufacturer instructions. After the appropriate incubation time, cells were washed 6 times with PBS-T for 15 minutes. Cells were then incubated for 1 hour at room temperature with the secondary antibody (goat anti-mouse IgG conjugated to Alexa 488; goat anti-rabbit IgG conjugated to Alexa 546) previously diluted in PBS-T containing 1% FBS and washed 6 times with PBS-T for 15 minutes. For nuclear staining, cells were lastly incubated for 10 minutes with Hoechst 33342 2 mM (Invitrogen) previously diluted in PBS-T, washed 3 times with PBS-T and coverslips were thereafter mounted on microscope slides using DAKO mounting medium (Glostrup, Denmark). They were later sealed with varnish, and preparations were observed in a Zeiss fluorescence microscope (Axiobserver Z1, Zeiss). For each experience various preparations were performed, and 3 coverslips per experimental condition were used for cell quantification. For each coverslip, 20 fields (exceptionally 40 fields, in some cases) were analysed using a 63x magnification. The ratio between iNOS, Iba-1, TH-positive cells and the total number of cells (as estimated by Hoechst 33342 labelling) was calculated and the results expressed as percentage of control or LPS 0.1 µg/mL, as described in the figure legends.

3.7. Phagocytosis Assay

To assess phagocytic activity, microglia cell cultures were incubated with 0.01% fluorescent microspheres (L1030-1L, Sigma) in M10C-G for 15 minutes in the incubator at 37 ºC. Cultures were then washed twice with MEM, to remove non-engulfed microspheres, and fixed with PFA 4% for 20 minutes. Lastly, cells were stained with Hoechst 33342 as described above for the immunocytochemistry. For each experience various preparations were performed, and 3 coverslips per experimental condition were used for cell quantification. For each coverslip, 20 fields were analysed using a 63x magnification. Results are presented as average of the ratio of total cells engulfing beads and the total number of cells as estimated by Hoechst 33342 labelling.

3.8. Nitric Oxide quantification

Nitric oxide (NO) production was assessed by quantification of total accumulated nitrite (NO^{2-}) in the experimental medium. Nitrite is a stable oxidation product of NO and one of the main products released into the medium. When nitrite reacts with sulphanilamide and N-1-naphthyl-ethylenediamine (NED), in acidic conditions, a coloured compound is formed. For such, at the end of the experiments, 50 µL of medium samples were taken and transferred to a 96 multiwell plate. Sulphanilamide solution (50 µL; 1% sulphanilamide and 5% H_3PO_4 in water) was added into the 96 multiwell plate and left shacking for 10 minutes, after which NED solution (50 µL; 0.1% N-1-napthylethylenediamine dihydrochloride in water) was added. Ten minutes later, absorbance at 550 nm was read in a microplate reader (xMark, Bio-Rad).

3.9. Measurement of reactive oxygen species production

The production of ROS by microglial cells was assessed using the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Sigma) as a ROS indicator. H_2DCFDA is a non-

fluorescent compound that is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. For such, one hour before the end of the experiments, cells were incubated with 100 µM H_2DCFDA dissolved in dimethyl sulfoxide (DMSO). Some wells were treated simultaneously with 12.5 µM hydrogen peroxide (H_2O_2) to be used as a positive control. Afterwards, fluorescence emitted was measured in a spectrofluorometer (Spectramax, Gemini XS, Molecular Devices LLC) using the excitation/emission wavelengths of 485/535 nm.

3.10. Data analysis and statistics

Results are shown as percentage of control or LPS 0.1 µg/mL and represent the mean ± S.E.M. of at least 4 experiments, as described in the figure legends. Statistical analysis was performed by the one-way ANOVA test followed by the Bonferroni Multiple Comparison Test. Values of $p < 0.05$ were considered statistically significant. Statistical analysis was performed using the program GraphPad Prism 6 (GraphPad Software, Inc.).

Chapter 4

Results and Discussion

4. Results and Discussion

4.1. Evaluation of Klotho's effect on LPS-induced microglial reactivity

As previously mentioned, microglia mediated inflammation is thought to be a major factor in the progression of NDD such as PD. In normal physiological conditions NO is an important second messenger, having a major role in intracellular signalling in the CNS. However, NO can be cytotoxic at high concentrations [100]. NO is produced by iNOS which is expressed by microglia. Under pathological situations its expression is augmented, resulting in high levels of NO that may be harmful for neurons [101]. Phagocytosis is another way by which microglia represent a hindrance to the DA viability, since when phagocyting microglia produce high levels of ROS [102]. As so, the control of microglial reactivity, which seems crucial in PD's development and progression, may prove to be a manner of interrupting the amplification of neuronal death [37]. Previous studies already pointed some anti-inflammatory properties of KL, such as reduction of pro-inflammatory markers in kidney [67, 84]. However, there are still no reports regarding its effects in brain inflammation.

In this work we aimed at assessing if KL is capable of modulating LPS-induced microglial reactivity by evaluating iNOS expression, NO release, phagocytic activity and ROS production. Primary microglia cultures were obtained from primary ventral midbrain glia cultures, from which astrocytes were removed by mild trypsinization.

Firstly, the minimal LPS concentration able to induce alterations in microglial reactivity markers was assessed. Therefore, microglia cultures were incubated with two different LPS concentrations (0.1 and 1 µg/mL) for 24 h, 5 days after astrocytes removal by mild trypsinization, and the number of iNOS-positive cells and NO release were determined and used as microglial reactivity markers in this optimization step.

As seen in Figure 4.1, LPS 0.1 µg/mL was able to induce a significant increase in the number of iNOS-immunopositive ($iNOS^+$) cells and NO production, and so we chose this LPS concentration in further assays using KL. To note, since iNOS levels in control condition were bellow detection limit, all the results were normalized for the LPS 0.1 µg/mL condition instead.

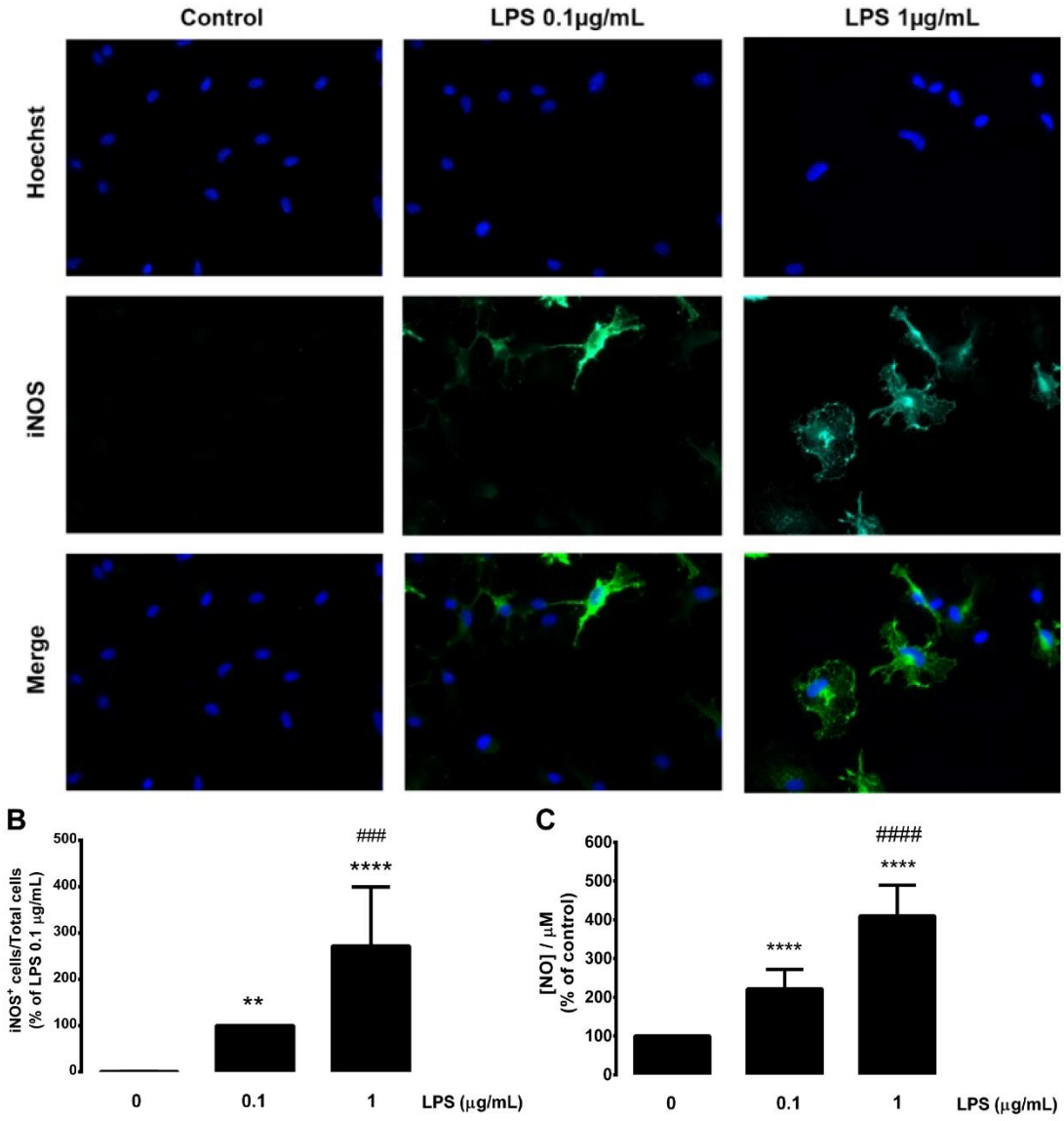


Figure 4.1 Optimization of LPS concentration able to induce microglial reactivity. Cells were exposed to 0.1 or 1 µg/mL LPS for 24 h. **A)** Representative images of the immunostaining for iNOS (green), in which nuclei were stained with Hoechst 33342 (blue). **B)** Quantification of iNOS-immunopositive cells ($iNOS^+$) for each condition, relative to the total number of cells, and expressed as percentage of the LPS 0.1 µg/mL condition. **C)** Quantification of NO released to the extracellular medium, expressed as percentage of the control condition (absence of LPS). Results are shown as mean ± S.E.M. of three independent experiences performed in triplicate. Statistical analysis was performed using one-way ANOVA test followed by Bonferroni's Multiple Comparison Test. (**$p < 0.01$ and ****$p < 0.0001$ compared to control; $^{###}p < 0.001$ and $^{####}p < 0.0001$ compared to LPS 0.1 µg/mL).

4.1.1 Klotho's effect on LPS-induced iNOS expression and NO release

Previous work from our group has shown that KL has a protective effect towards DA neurons, when aplied prior to 1-methyl-4-phenylpiridinium (MPP^+) exposure, which is mediated by the presence of glial cells (unpublished results). As so, this work aims at evaluating if KL may have an effect on microglial cells' reactivity. For such, primary microglia cultures from the ventral midbrain were treated with KL at a concentration of 0.4 µg/mL for 24 h or 1 h prior, or 1 h after the exposure to LPS 0.1 µg/mL for aditional 24 h. Looking at the obtained results (Figure 4.2), we can see that KL is able to significantly

prevent both LPS-induced increase in iNOS expression and NO release. However, while NO release is maintained at levels close to the control situation by the KL incubation, iNOS expression is still significantly different from control levels. This may indicate that, although being expressed, iNOS could be inhibited by KL leading to NO release similar to the one obtained under control conditions. As opposed to other forms of NOS which regulation is calcium-dependent, iNOS has been described as calcium-insensitive [103]. Indeed, iNOS is mainly regulated at the transcriptional level, but also at posttranscriptional, translational and postranslational levels through effects on protein stability, dimerization, phosphorylation, cofactor binding and availability of substrates (oxygen and L-arginine) [104]. In our case, iNOS is likely being regulated at the posttranscriptional level, since there still exist an increase in iNOS expression but no increase in NO production. KL treatment 1 h after LPS exposure reduced iNOS expression, suggesting that LPS-induced inflammation may be reverted. However, since the time gap between the beginning of LPS exposure and the application of KL is relatively short, inflammation was most likely not yet established and, as so, larger time gaps between LPS and KL application need to be evaluated to allow the establishement of inflammation and trully acess recovery from inflammation.

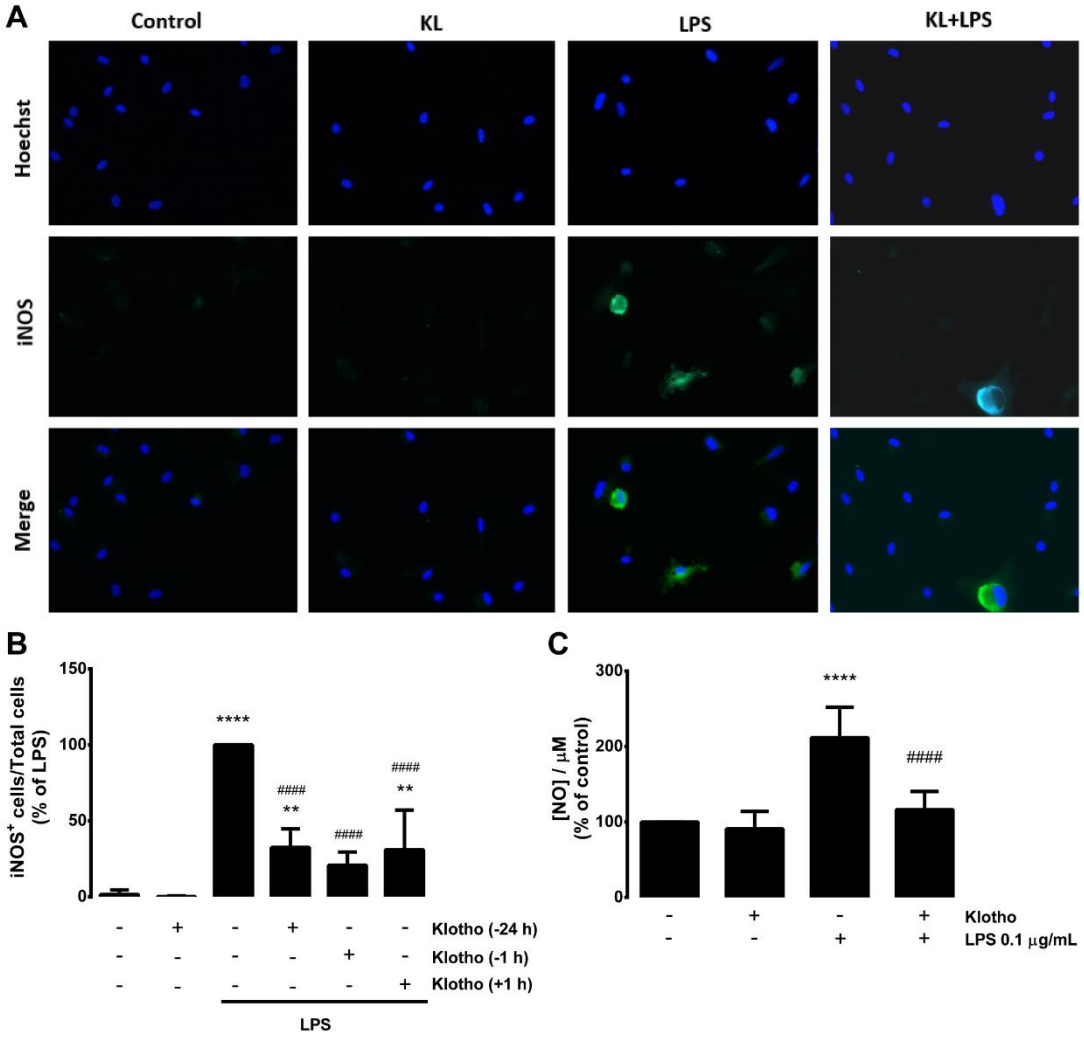


Figure 4.2 Effect of KL on LPS-induced iNOS expression and NO release. Cultures were treated with 0.4 µg/mL KL for 24 h or 1 h, and then exposed to LPS 0.1 µg/mL for an additional period of 24 h, or treated with 0.4 µg/mL KL after 1 h of LPS application. **A)** Representative images of the immunostaining for iNOS (green), in which nuclei

were stained with Hoechst 33342 (blue). KL+LPS representative images are from a KL preincubation of 24 h. Only one condition of KL treatment and LPS exposure is shown as the three KL incubation times showed similar results. **B)** KL's effect on the LPS-induced increase in the iNOS-immunopositive ($iNOS^+$) cell number, relative to the total number of cells, and expressed as a percentage of control (no KL and no LPS). **C)** Effect of KL preincubation (24 h) on LPS-induced NO release. Data are shown as mean ± S.E.M. of 4 independent experiences performed in triplicate. Statistical analysis was performed using one-way ANOVA test followed by Bonferroni Multiple Comparison Test. (**$p < 0.01$ and ****$p < 0.0001$ compared to control (no KL and no LPS); $^{\#\#\#\#}p < 0.0001$ compared to the LPS condition).

4.1.2 Klotho's effect on LPS-induced phagocytic activity

To assess microglia's phagocytic activity, cells were exposed to fluorescent microspheres after 24 h KL incubation followed by 24 h incubation with LPS. The cells that incorporated the microspheres were considered phagocytic (Figure 4.3). We observed that LPS exposure increased microglial phagocytic activity by nearly 75%, with KL pre-incubation preventing this LPS-induced phagocytosis and maintaining microglial phagocytic activity closer to control values. Although phagocytosis may be important for clearance of cellular debris and pathogens it has also been shown that when microglia is phagocyting it also produces high levels of ROS which cause neuronal degeneration [102]. As so, it was of interest to determine ROS production in our cultures and the results are presented in section 4.1.3.

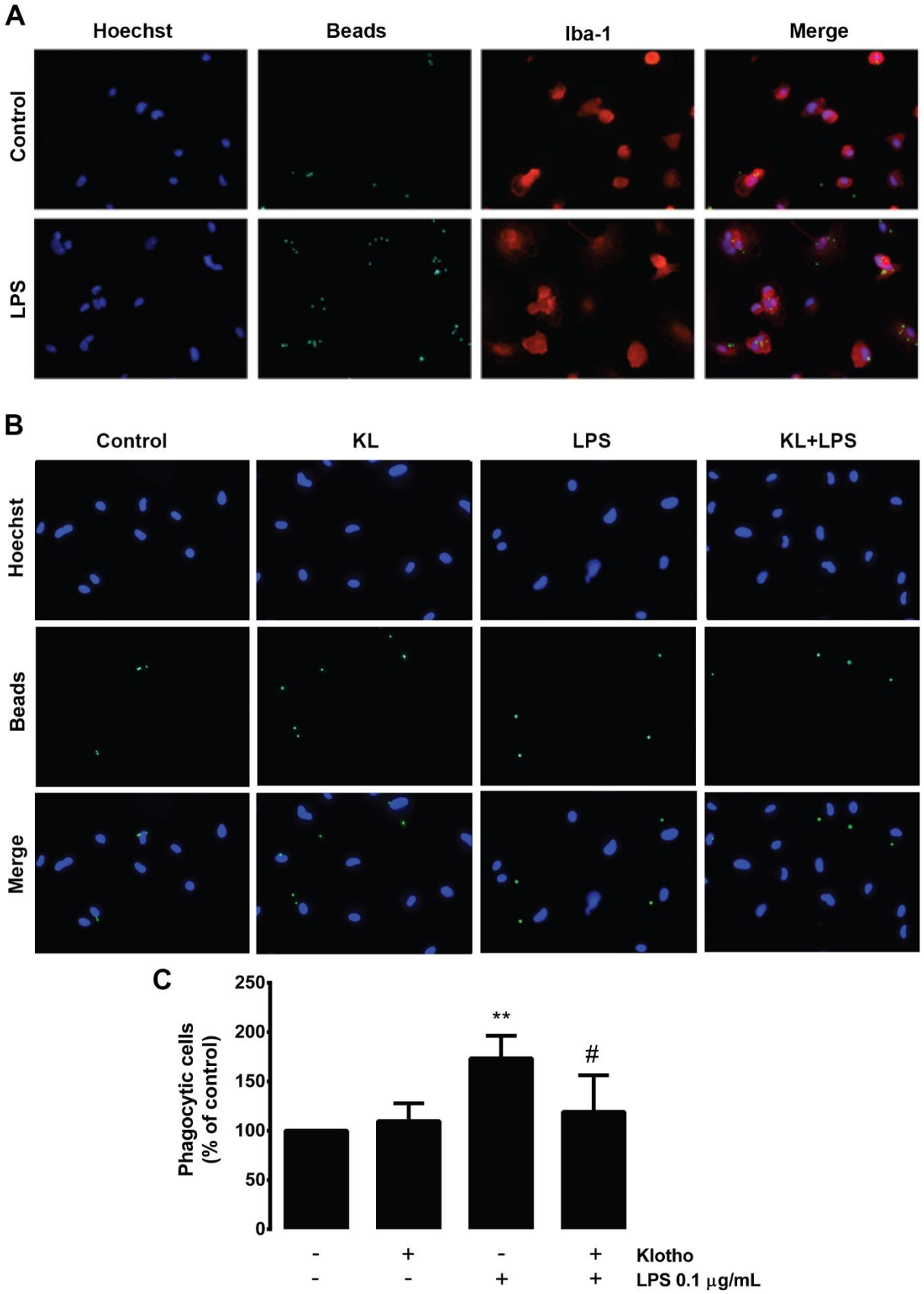


Figure 4.3 Effect of KL on microglial phagocytic activity. Cultures were treated with 0.4 μg/mL KL for 24 h, and then exposed for another 24 h to LPS 0.1 μg/mL. **A)** Representative fluorescence images of ventral midbrain microglial cultures exposed to microspheres (green), in which microglia were marked with Iba-1 antibody (red) and nuclei stained with Hoechst 33342 (blue) **B)** Representative fluorescence images of ventral midbrain microgial cultures

exposed to fluorescent microspheres (green), in which nuclei were stained with Hoechst 33342 (blue). **B)** Quantification of cells that engulfed the fluorescent microspheres after 24 h incubation with LPS, in the presence or in the absence of KL preincubation for 24 h. Results are presented as the number of phagocytic cells, relative to the total number of cells, and expressed as a percentage of control (no KL and no LPS). Data are shown as mean ± S.E.M. of 4 independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA test followed by Bonferroni Multiple Comparison Test. (**$p < 0.01$ as compared to control; $^{\#}p < 0.05$ as compared to the LPS condition).

4.1.3 Klotho's effect on LPS-induced ROS production

Firstly, it was important to find an exposure time with LPS 0.1 µg/mL that would significantly and maximally increase ROS production in our microglia cultures. We found that at 12 h of LPS 0.1 µg/mL incubation (Figure 4.4) there was a maximum in the production of ROS by microglial cells (increase of 130%). Therefore, this incubation time was selected to study the effect of KL in LPS-induced ROS production, as described below.

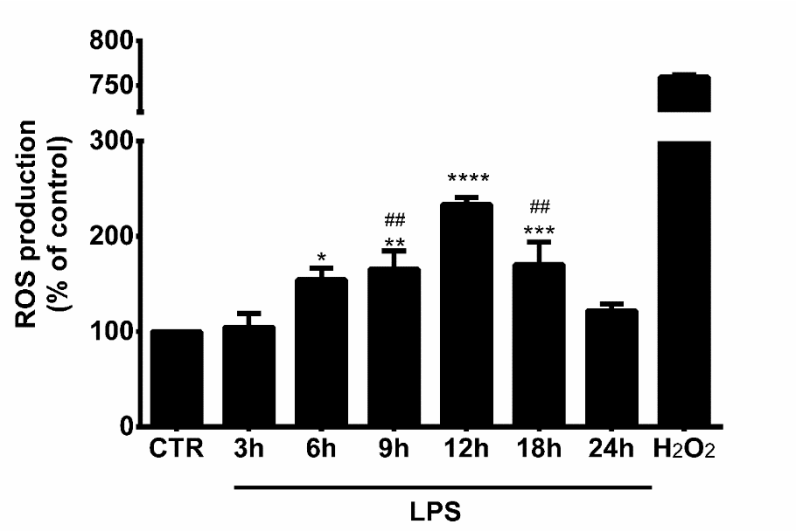


Figure 4.4 Optimization of LPS exposure time able to induce maximal ROS production. Microglial cells were exposed to 0.1 PS for 3, 6, 9, 12, 18 or 24 h, and incubated with H_2DCFDA 1 h before fluorescence readings. Control (CTR) cells only received H_2DCFDA (no LPS incubation), and some wells received H_2O_2 as a positive control at the same time as H_2DCFDA. Data are shown as mean ± S.E.M. of 3 independent experiments performed in 6 replicates. Statistical analysis was performed using one-way ANOVA test followed by Bonferroni Multiple Comparison Test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ when compared to control (no LPS); $^{\#\#}p < 0.01$ when compared to 12 h exposure).

Therefore, to assess KL's effect on LPS-induced ROS production by microglia, cells were pre-incubated with KL for 24 h or 1 h and later exposed to LPS 0.1 µg/mL for 12 h. Previous work from our group (unpublished results) had found that KL was able to reduce MPP^+-induced ROS production in neuron-glia cultures, being more effective for shorter pre-incubation times (1 h prior to MPP^+ exposure). Therefore, we evaluated KL's effect in LPS-induced ROS production with pre-incubation times of 24 h, for which we had observed a preventive effect regarding phagocytosis, and 1 h, following previous results from our group.

Surprisingly, and although there was no statistical difference, it seems KL could not prevent the LPS-induced increase in total ROS production neither for 24 h nor 1 h pre-incubation times (Figure 4.5), even though KL has been widely reported in the literature as an anti-oxidative protein [96, 105]. Even

more, as previously stated, a co-link between phagocytosis and ROS production has been reported but that was not visible in our case where KL was able to prevent phagocytic activity but unable to prevent ROS production. As so, we can conclude that although not being able to modulate all the studied parameters, KL pre-incubation is able to modulate microglia reactivity as seen through iNOS expression, NO production and phagocytosis prevention which have been pointed as key indicators of microglial reactivity [106].

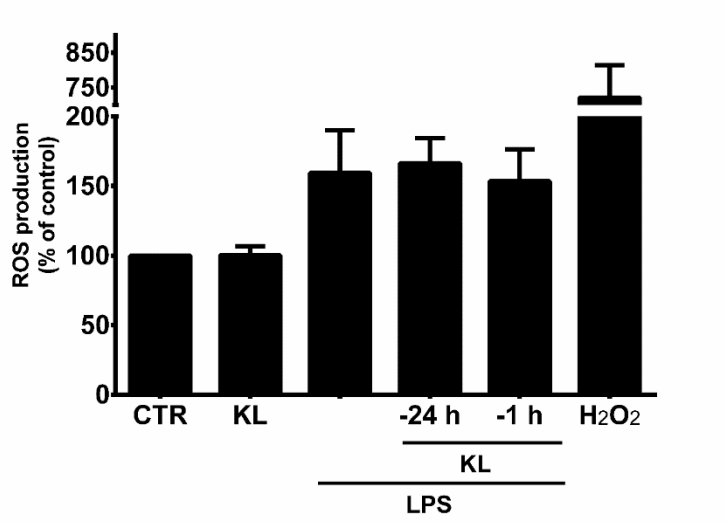
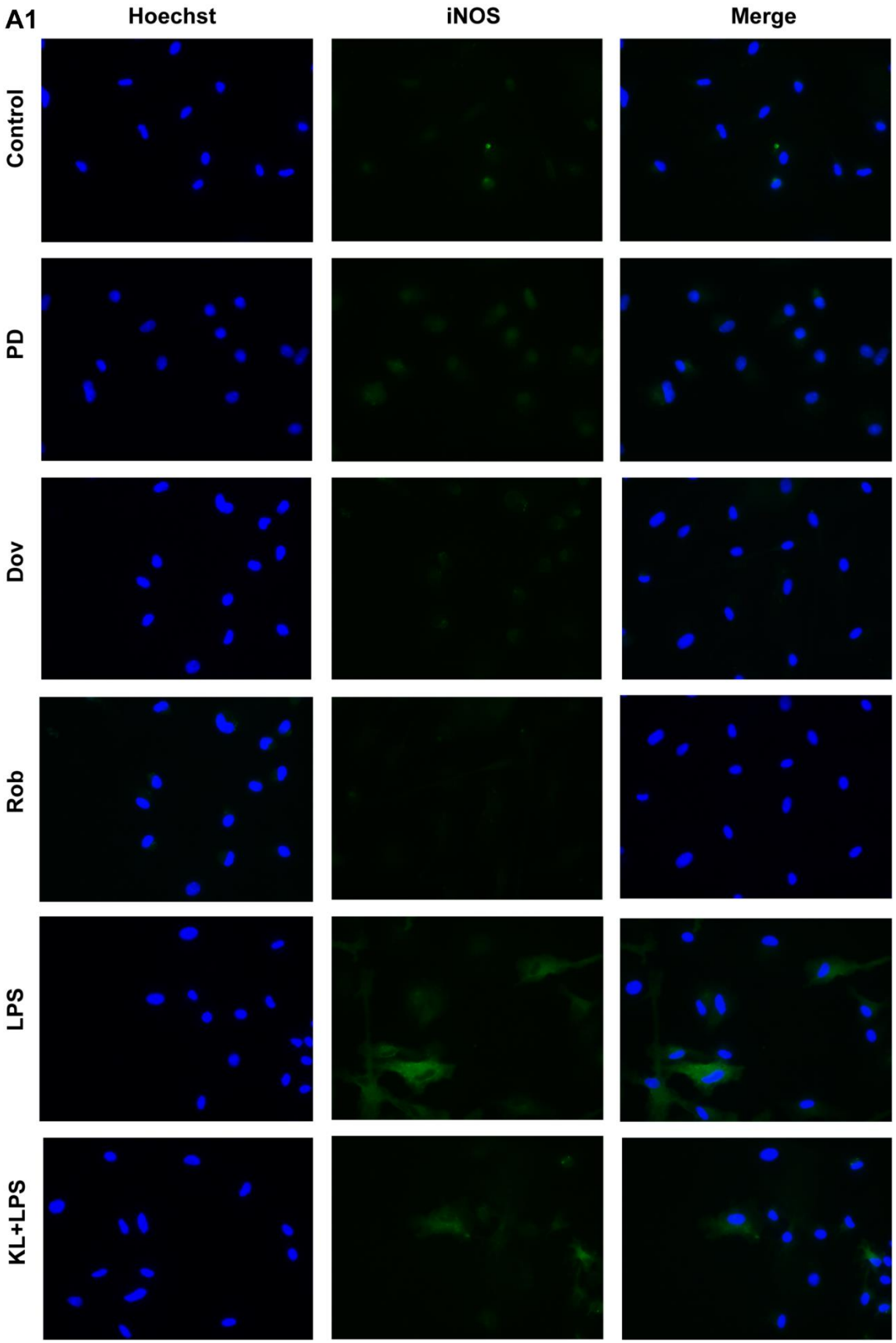


Figure 4.5 KL pre-incubation had no effect on LPS-induced ROS production. Microglial cells were treated with 0.4 µg/mL KL for 24 h or 1 h, and then exposed for another 12 h to LPS 0.1 µg/mL. H_2DCFDA was applied 1 h before the readings, and some wells received H_2O_2 as positive control at the same time of H_2DCFDA. Data are shown as mean ± S.E.M. of 3 independent experiences performed in 6 replicates. Statistical analysis was performed using one-way ANOVA test followed by Bonferroni Multiple Comparison Test.

4.2 Receptors mediating Klotho's anti-inflammatory effect

Another objective of this work was to explore possible receptors mediating KL's anti-inflammatory effect. FGFRs had been pointed as possible receptors mediating KL's effects [78], being the isoforms 1, 3 and 4 the ones reported to be present in microglial cells [107]. As so, we used specific inhibitors for these receptors and evaluated if its presence would affect KL's ability to modulate microglia reactivity, namely LPS-induced iNOS expression (Figure 4.6). Unfortunately, it was only possible to perform one experiment (n=1) for this objective, but the results suggest that the contribution of FGFR3 should be further explored in order to clarify its involvement for KL's ability to modulate microglia reactivity. When cells were pre-treated with Dovitinib, a selective FGFR3 inhibitor, there were around 13% $iNOS^+$ cells while when cells were not treated with any inhibitor or were pre-treated with PD166866 or Roblitinib (respectively FGFR1 and FGFR4 specific inhibitors) there were about 3% $iNOS^+$ cells.

A previous *in vivo* study reported that hippocampus microglia was activated by the sole inhibition of the FGFRs [108]. However, the same was not true for our experiment, which may possibly be derived from the lack of other cell types in the culture or by regional differences in microglia. Still, what is presented in this thesis is the result of a single experiment and further experiments should be performed regarding this objective so conclusions can be drawn regarding the contribution of FGFRs to the anti-inflammatory effect of KL in ventral midbrain microglia.


A1
Hoechst
iNOS
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Control
PD
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Rob
LPS
KL+LPS

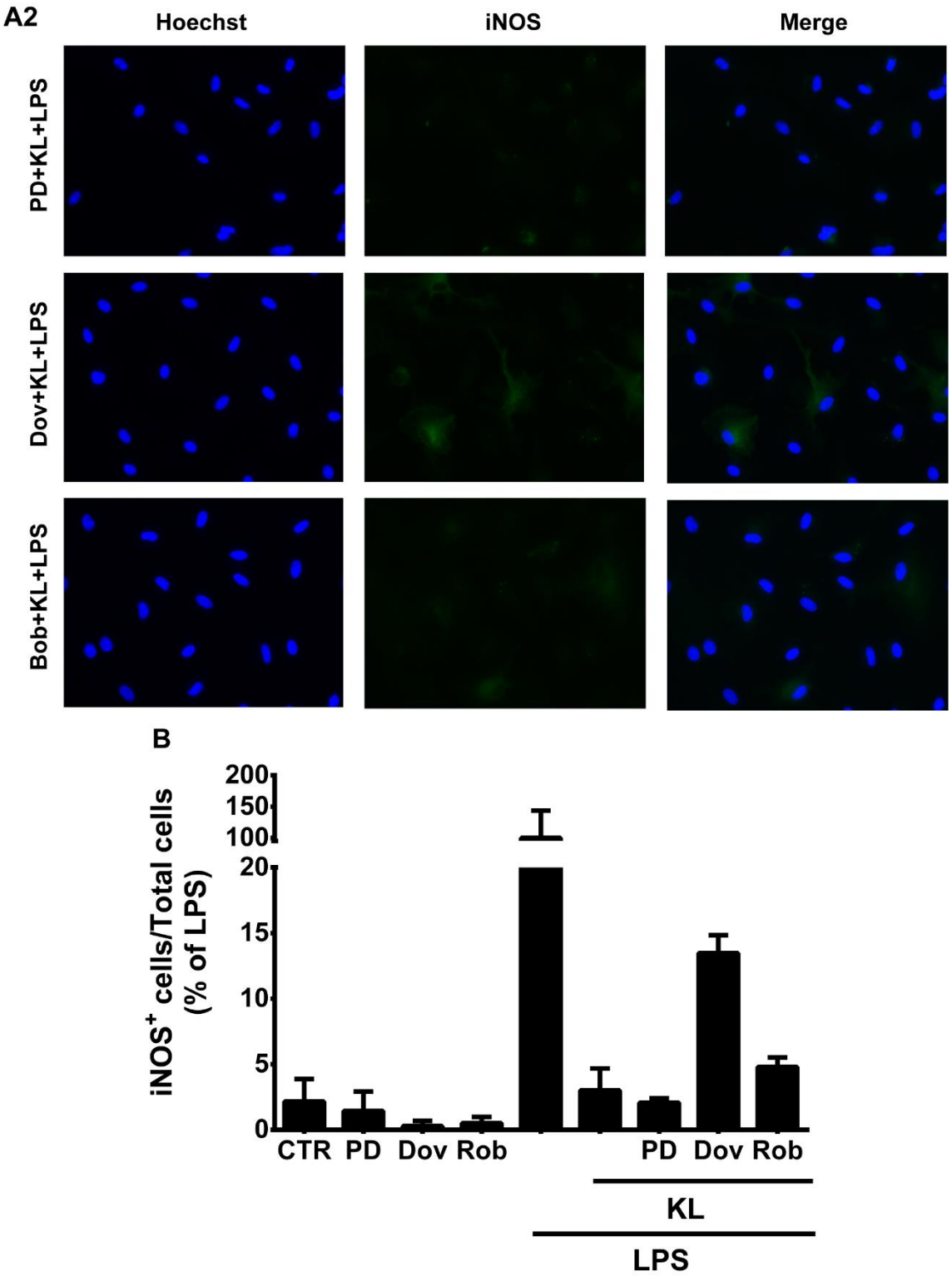

Figure 4.6 Effect of FGFRs inhibitors (PD166866, Dovitiniv and Roblitinib) on LPS-induced iNOS expression after preincubation with KL for 24 h. Cells were treated with 0.5 µM for PD166866 or 1 µM for Dovitinib and Roblitinib. One hour later 0.4 µg/mL KL was applied and 24 h later cells were exposed to 0.1 µg/mL LPS for an additional period of 24h. Control cells (CTR) did not receive any treatment. **A)** Representative images of the immunostaining for iNOS (green), in which nuclei were stained with Hoechst 33342 (blue). **B)** FGFRs inhibitors (PD; PD166866, Dov; Dovitinib, Rob; Roblitinib) effect on KL's anti-inflammatory effect regarding iNOS expression. Data are shown as mean ± S.E.M. of 1 experiment performed in triplicate.

4.3 Relevance of Klotho's anti-inflammatory effect on DA neuronal protection

Determined that KL is able to modulate microglial reactivity, namely by preventing LPS-induced iNOS expression, NO production and phagocytosis, the next step was to assess if that would be a contributing factor for the ability of KL to protect DA neurons from MPP^+-induced toxicity, in neuron-glia mixed cultures, as previously shown by our research group (unpublished results). For such, we wanted to obtain primary cultures that had both glial cells and neurons, with DA neurons among them, but deprived of microglial cells. As so, we obtained primary ventral mesencephalic cultures from Wistar embryos, which contained microglia, astrocytes and neurons, and depleted microglia from the cultures. Based on the literature, there are several microglia depletion procedures using L-leucine methyl ester (LME), a lysosomotropic agent which is converted to a membranolytic compound by dipeptidyl peptidase I that selectively kills phagocytic cells, such as microglia [109, 110]. Unfortunately, there is no report of LME treatment in our cultures of interest. The available protocols, from other brain regions' cultures had differences regarding both exposure time and concentration of LME, but what was mostly reported with success were treatments of 1 h with concentrations ranging between 25 and 75 mM, above which it was reported to be toxic to astrocytes [111]. Therefore, our mesencephalic neuron-glia mixed cultures were treated with three different concentrations of LME (25, 50 or 75 mM) for 1 h, and then washed with PBS 1x in order to remove dead cells.

Looking at the results, we can see that LME treatment didn't affect DA neurons viability, even for the higher concentration used (75 mM; Figure 4.7). However, microglia depletion was far from what was expected and reported for cultures obtained from other brain regions, as well as far from what would be desired for the aim of our experiments. We obtained around 50% microglial depletion (Figure 4.7), whereas nearly total microglia depletion was reported in the literature for other cell culture models [111]. Such a great difference in microglia depletion in our cultures compared to other reports may be due to regional differences of microglia [112], which may be granting mesencephalic microglia as more resistant to LME exposure. In this work we didn't evaluate LME effect on astrocytes' viability since previous reports in the literature stated that for the used concentrations no changes in astrocytes viability or function was observed [111].

Hence, since microglial depletion was not satisfactory in these cultures, another member from our group deepened more into this topic and assessed if MPP^+ exposure, which selectively decreases DA neurons viability, would lead to microglial activation and if KL would be able to modulate its reactivity, while protecting DA neurons from MPP^+ toxicity. MPP^+ uses dopamine transporters and once inside the DA neurons it blocks the electron transport chain by inhibiting the mitochondrial complex I, and leading to severe energy dysfunctions, ROS production, oxidative damage to the membrane and activation of apoptotic cascades [113]. The results indicated that in our mesencephalic neuron-glia mixed cultures, MPP^+-induced toxicity of DA neurons was not paralleled by an increase in microglial reactivity, suggesting that this in vitro model would not allow us to infer about the contribution of KL's anti-inflammatory effect on its ability to protect DA neurons. Consequently, a different model that allow us to see degeneration of DA neurons and microglial reactivity in the same culture, with microglial reactivity being the cause of DA degeneration or exacerbating it as it happens in *in vivo* models, will be needed

in order to assess if microglial modulation by KL would indeed be relevant in preventing the DA neurodegeneration process.

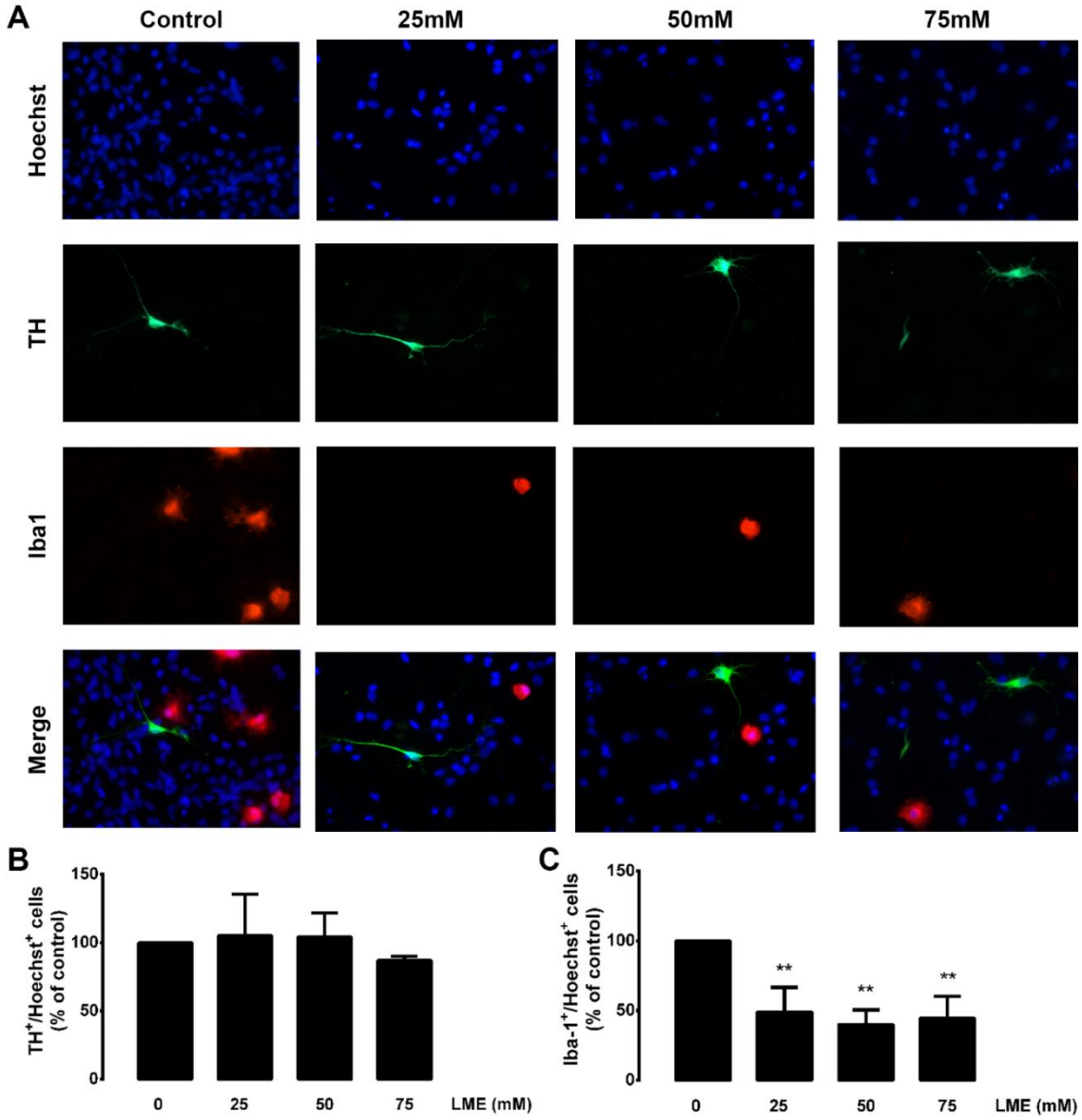

Figure 4.7 Dopaminergic viability and microglial depletion with LME treatment. Mesencephalic neuron-glia mixed cultures were treated with 25, 50 or 75 mM LME for 1 h and allowed to rest with fresh NBM for 24 h. **A)** Representative images of the immunostaining for TH (DA neuron marker, green) and Iba1 (microglial marker, red), in which nuclei were stained with Hoechst 33342 (blue). **B)** Dopaminergic viability after LME treatment, as determined by the TH-immunopositive (TH^+) cell number, relative to the total number of cells ($Hoechst^+$), and expressed as a percentage of control (absence of LME). **C)** Microglial viability after LME treatment, as determined by the Iba1-immunopositive ($Iba\text{-}1^+$) cell number, relative to the total number of cells ($Hoechst^+$), and expressed as a percentage of control (absence of LME). Data are shown as mean ± S.E.M. of 3 independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA test followed by Bonferroni Multiple Comparison Test. (**$p < 0.01$ as compared to control).

Chapter 5

Conclusions

5. Conclusions

In this work we assessed that KL is able to modulate microglial reactivity, as seen through iNOS expression, NO production and phagocytic activity. We aimed to identify the possibility of FGFRs being involved in the anti-inflammatory effect exerted by KL but additional experiments should be performed so clear conclusions can be drawn. Even so, among those present in microglia, FGFR3 may be a promising receptor mediating KL's anti-inflammatory effect. It was also of our interest to assess if the KL anti-inflammatory effect would be impactful in preservation of DA neurons but were unable to obtain a mixed culture deprived of microglia cells. Even more, a colleague verified that in the MPP^+ model of DA neurons degeneration no microglial reactivity was occurring, and so another model where DA neurons degeneration and microglial reactivity are simultaneously present, possibly with microglial reactivity being the cause of DA neurons degeneration, will be needed to evaluate the relevance of KL's anti-inflammatory effect for DA neuroprotection also mediated by this anti-ageing protein.

Chapter 6

References

6. References

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