Unraveling molecular signaling in neurodegenerative diseases
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CHAPTER 1
General introduction
Neurodegenerative diseases

Neurodegenerative diseases represent a main threat to human health. These age-dependent disorders are becoming increasingly prevalent, in part because the elderly population has increased in recent years [1,2]. Neurodegeneration is characterized by a progressive loss of neurons that reside in brain areas associated with motor, sensory, cognition and perceptual functions. Therefore, cognitive and behavioral deficits are highly attributed to progressive neural cell death in the central nervous system (CNS) [3,4]. Differences in origin and the role of both genetic and environmental factors in the onset and progression of neurodegenerative diseases entangle our understanding of the involved pathogenic mechanisms. Accumulation of misfolded proteins form intracellular inclusions or extracellular aggregates in particular brain regions and are considered the main pathological hallmarks of many neurodegenerative diseases [5].

Alzheimer disease

Alzheimer’s disease (AD) is a multifactorial and heterogeneous disease; it can be either familial or associated with a mutation of an autosomal dominant gene in approximately 5% of cases, and sporadic in 95% of the remaining cases. A common cause is not known yet, and the possible factors that contribute to the development of the disease are still being investigated. It has been suggested some risk factors that may be associated to the development of the disease, such as age, gender, family history, education, hypertension, diabetes, high cholesterol, depression, low cognitive and physical activity, lifestyles and medications. However, the mechanism by which these risk factors contribute to the pathogenesis of AD has not been clearly established [6,7].

AD is a neurodegenerative process that exhibit a progressive deterioration of the brain, initially disturbing the temporal lobe and the hippocampus producing memory problems. Later, the parietal lobe is affected, which involves loss of spatial visualization processes, knowledge of habits and uses, and finally the frontal lobe is damaged causing changes in personality. These events in the brain are reflected in the symptomatology of the disease that also includes attention problems and spatial orientation, language difficulties, unexplained mood swings, erratic behavior and loss of control over bodily functions, generating dependency and inactivity of patients. However, AD does not affect all patients in the same way, these symptoms vary in severity and chronology, fluctuations are reported even daily with the superposition of symptoms [8,9].

Neuropathology of AD is characterized by a widespread accumulation of neuritic plaques and neurofibrillary tangles composed of deposits of beta-amyloid peptide (βA) and abnormally hyperphosphorylated tau protein (phospho-tau) respectively. These aggregates start to appear in the pyramidal cells of the cortex and hippocampus and they start to spread out to other regions of the brain causing neuronal disconnection. There are other cellular alterations, such as the decrease in the neurotransmitter Acetylcholine (Ach), synaptic loss, inflammation and neuronal cell death [10,11].

Lipids in AD

Lipids are a diversified and ubiquitous group of biomolecules which have several relevant biological functions, such as storing energy, second messenger in cell signaling, and acting as structural components of cell membranes [12]. By regulating the chemical and mechanical properties of membranes, lipids influence vesicle fusion and fission processes, ion flux, and lateral diffusion of membrane proteins [13].

Lipids can be classified based on their composition. As described by Fahy et al., 2011, lipids are broadly classified into simple lipids (esters of fatty acids with alcohol); these include fats, waxes), complex lipids (esters of fatty acids with alcohols containing additional groups such as phosphate, nitrogenous base, carbohydrate, protein etc.; these include phospholipids, glycolipids, lipoproteins, sulfolipids), and derived lipids (derivatives obtained on the hydrolysis of simple and complex lipids which possess the characteristics of lipids; these include isoprenoids, steroids, ketone bodies, fatty acids and caretonoids [12,14] as we can observe in Figure 1.

Growing evidence supports the influence of lipid changes in the process of normal cognitive aging and the etiology of age-related neurodegenerative diseases [15]. For example, higher midlife serum cholesterol increases AD risk and impairs late-life cognition [16,17]. Cholesterol and sphingolipid-enriched membrane microdomains called “lipid rafts” can modulate the amyloidogenic processing of APP leading to altered βA aggregation [18].

Human apolipoprotein E (ApoE) is essential in lipid metabolism and cholesterol transport in plasma and several tissues. Dupuy et al., 2001 described that ApoE is synthesized in the CNS and is recognized as the major lipid carrier protein in the brain. Among several member of the ApoE family, ApoE4 has emerged as a significant genetic risk factor for vascular disease

![Figure 1. Classification of lipids. Modified from Bailwad et al., 2014 (14)](Image 539x69 to 913x173)
and familial and sporadic late-onset AD\textsuperscript{[19]}, ApoE4 is implicated in senile plaque formation by its affinity for βA peptide leading to insoluble complexes and in turn amyloidogenesis. ApoE4 can also join to tau and microtubule-associated proteins (MAP), and thus be implicated in the development of neurofibrillary tangles underling to ApoE4 in the highest genetic risk factor for late-onset AD\textsuperscript{[20]}.

**Phospholipids in AD**

Kosicek and Hecimovic, 2013 describe phospholipids as structurally and biologically important molecules, which form cellular membranes and are involved in the behavior of membrane proteins, receptors, enzymes and ion channels intracellularly or at the cell surface. Since the brain is one of the richest organs in lipid content, changes in the brain phospholipid levels could lead to different pathogenic processes. Different regions of the brain differ in phospholipid composition\textsuperscript{[21]}. Phospholipids consist of two long chains, with non-polar acyl fatty groups joined to small polar groups including a phosphate (Figure 2). Phospholipids together with cholesterol and glycolipids represent around 50 to 60% of total membrane lipids, playing a very critical role in the physical properties of the lipid bilayer\textsuperscript{[22]}.

Publications from late 1980 and 1990 suggested that decreased and alterations in brain phospholipid metabolism could be connected with AD\textsuperscript{[21]}. Increase in the activity of PLA\textsubscript{2} isofoms and lysophospholipases elevation in phosphodiesters, phosphomonooesters, fatty acids, prostaglandins, isoprostanes, 4-hydroxynonenals, and other lipid mediators has been reported in AD\textsuperscript{[23]}.

![Figure 2. Structure and main roles of phospholipids in neural membranes](image)

A reduction of Phosphatidylinositol (PI) levels\textsuperscript{[24]} and phosphatydilethanolamine (PE) levels were found in post-mortem brain samples from individuals with AD compared to controls\textsuperscript{[23,26]}. In parallel, a decreased of phosphatydilcholine (PC) or unchanged PC levels\textsuperscript{[21,25]} have been reported. Also, levels of Lyso-phosphatidylcholine (LPC) in cerebrospinal fluid (CSF) of AD patients were reduced compared to controls. Interestingly, in an early stage of AD, brain levels of PC, PI and PE in both white and gray matter were unchanged\textsuperscript{[21]}. These alterations in AD indicates relevant changes in the metabolism of phospholipids in the brain that may be closely associated with membrane alterations and damage in AD (Figure 3)\textsuperscript{[27]}.

**Current therapy in Alzheimer’s disease**

Available drugs for the treatment of AD use the principle of inhibition of AchE (acetylcholinesterase). This enzyme hydrolyzes and inactivates Ach (acetylcholine), a main neurotransmitter in the neuronal communication of the nervous system that is reduced in AD. Inhibitors of AchE increase Ach in the synaptic junction, and this helps to improve cognitive function\textsuperscript{[28]}.

![Figure 3. Principal molecular mechanism associated with lipid mediator-mediated neurodegeneration. AA, arachidonic acid; Aβ, β-amyloid; ADAM, A Disintegrin And Metalloprotease; sAPPα, soluble amyloid precursor protein α; sAPPβ, soluble amyloid precursor protein β; APP, amyloid precursor protein; BACE1, β-site APP cleavage enzyme; CERase, ceramidase; 4-HNE, 4-hydroxynonenals; IsoP, isoprostane; COX-2, cyclooxygenase 2; cPLA\textsubscript{2}, cytosolic phospholipase A\textsubscript{2}; DHA, docosahexaenoic acid; 15-LOX, lipoxigenases; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; NMDAR, N-methyl-D-aspartate glutamate receptor subtype; PC, phosphatidylcholine; PE, phosphatidylethanolamine; ROS, reactive oxygen species; p75-NTR, p75 neurotrophin receptor; SM, sphingomyelin; SMase, sphingomyelin phosphodiesterase; Sph, sphingosine; SphK, sphingosine Kinase; Sph-1P, shingosine 1 phosphate. Figure from Frisardi et al, 2011\textsuperscript{[15]}](image)
Drugs that elevate the levels of ACh as the galantamine, donepezil and rivastigmine are indicated in the first stages of the disease to delay the deterioration of memory and attention. These treatments are combined with others that act in a symptomatic level for depression, sleep disturbances, or complications as constipation, incontinence, dehydration, urinary infections and ulcers caused mainly by immobility or thrombophlebitis. However, all these drugs have numerous side effects altering the function of the gastrointestinal tract by inducing diarrhea, loss of appetite, nausea, vomiting, weight loss and hepatotoxicity, but also they can lead to fatigue, insomnia, and muscle cramps that encourage the search for new therapeutic targets.

Immunotherapy for AD treatment was considered with AN-1792 vaccine. This vaccine enhanced the production of βA antibodies in the serum stimulating the immune system to eliminate βA plaques and preventing the formation of new plaques. However, adverse effects such as meningoencephalitis have been reported, resulting in discontinuation of the treatment. Currently, there are immunotherapies in different clinical phases, which evaluate new strategies to decrease βA, or at least for slowing down the progression of the disease.

Among other medications, one of the most used is memantine, a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) receptors, to which it binds with a moderate affinity. This medication improves the cognitive performance and functioning of patients with moderate to severe AD, however, it continues to have a palliative function in this disease.

Neuroprotection by natural products in AD

There has been a recent explosion of interest in natural products and their potential multifunctional effects on AD and other neurodegenerative diseases. We already report studies have reported that the oral administration of some flavonoids (apigenin, EGCG, rutin, myricetin, resveratrol, quercetin and fisetin) to mice prevents the development of AD pathology by inhibiting various βA aggregation pathways and thus increases their ability to solve memory tasks. These effects may be mediated by the activation of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF), which are involved long-term potentiation (LTP) and it has consequences in learning processes. Furthermore, our group demonstrated that the intraperitoneal administration of quercetin in an old triple-transgenic AD mice model for three months reduces the C-terminal fragment (CTF) cleavage of Amyloid precursor protein (APP), production of βA1–40 and βA1–42, and βA plaque immunoreactivity in central regions affected by this disease. Additionally, quercetin significantly decreases the hyperphosphorylation of tau in old 3xTgAD mice, which correlated with the recovery of memory.

Given the diverse etiological nature of AD, many neural targets that can be addressed. The majority of natural products have several targets, strategies such as prophylactic treatment may help improve the potency of existing drugs and aid in the development of new therapies. For example, cocktails comprising approved drugs with natural products could be considered as standard therapies for AD.

Cerebrovascular diseases

Cerebrovascular diseases (CVD) are the third cause of death in the world and the second in Latin America after 45 years old according to the Pan American Health Organization. Additionally, it is reported as the first cause of permanent disability in adulthood, as many of the surviving patients suffer substantial sequelae that limit their activities in daily life. Its morbidity and mortality not only cause suffering to patients and their families but also entails a high social and economic cost.

Cerebral strokes can be divided into ischemic and hemorrhagic, with an incidence of 84 and 16%, respectively. Ischemic stroke occurs when a blood vessel carrying blood to the brain is blocked by a blood clot and is characterized by a decrease or interruption of blood flow in one area of the brain. Hemorrhagic stroke is caused by the rupture of a blood vessel, either in the parenchyma or in the brain surface; blood spills into or around the brain and creates swelling and pressure, damaging cells and tissue in the brain.

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)

CADASIL is the most common monogenetic cause of adult-onset progressive cerebrovascular disease. The disease results from mutations in the NOTCH3 gene, a 34-exon gene located on chromosome 19p13.2-p13.1. NOTCH3 encodes a transmembrane protein involved in cell signaling and differentiation and a transmembrane receptor primarily expressed in systemic and intracranial vascular smooth muscle cells.

CADASIL generally affects young people, and the first ischemic strokes occurs between 30 and 66 years of age. Clinical symptomatology is very variable even within individuals of the same family, the disease is commonly progressive. On average, it leads to the inability to walk without assistance between 56 and 64 years, restriction to bed between ages 59 and 69 years, and age of death between 61 and 74 years. Quality of life from patients is fragile due to recurrent strokes, severe migraines with aura, mood changes, apathy, and epilepsy that produce cognitive impairment, along with distinctive imaging findings, usually precede clinical strokes by years to decades.
Ischemic stroke

Ischemic stroke is initiated by a constriction of the blood flow to the brain leading to immediate deficit of nutrients and oxygen that are normally required for the maintenance of the brain’s metabolic requirements. If restoration of perfusion occurs very early after the onset of ischemia, this can decrease the damage from stroke, but the efficacy of reperfusion is restricted by secondary injury mechanisms [53,54]. When an arterial occlusion occurs, the subsequent ischemia is not homogeneous throughout the affected zone. Instead it is a dense ischemic central nucleus called ischemic core with severe compromise of cerebral blood flow (CBF), producing high cell death by necrosis. Ischemic core is surrounded by a perimeter of moderate ischemic tissue called “penumbra” where the cellular metabolism and viability is sometimes preserved but has impaired electrical activity [55]. Ischemic penumbra has a variable outcome, and tissue rescue may be reached when reperfusion is initiated within the first 6 hours following the insult. The third region is known as the extra penumbra zone, peri-infarct or zone of oligemia (Figure 4), in which the blood flow is higher than 40% and tissue is completely vital [56].

In conditions of cerebral ischemia, the cells of the affected area quickly use their reserves of glycogen and increase lactate production through anaerobic glycolysis, causing tissue acidification. Besides, there is a substantial reduction in the concentration of ATP, which alters the transmembrane ion gradients and the loss of ionic homeostasis leading to an intracellular increase of sodium and calcium ions [56,58]. Thus, the accumulation of intracellular calcium has been established as the critical step in neuronal death by triggering the activation of proteases, lipases, DNases, and calpains, promoting lysis of structural proteins. At the same time, the presence of extracellular calcium increases the release of glutamate, a toxic neurotransmitter that contributes to cell death [50–52].

One of the most sensitive parameters in the reduction of blood flow is the inhibition of protein synthesis during ischemia. Polyribosomes remain aggregated and stop the synthesis of some proteins but is recognized that levels of proteins involved in heat shock protein (Hsp) are increased [64]. If these conditions are prolonged for a long time will produce a deficit in essential proteins that allow cell survival [64]. Besides, inflammation is produced in the vascular endothelium, thickening of the astrocytic feet. These cause alterations in the matrix–integrin interactions, leukocyte-endothelial cell adhesion, platelet activation, leukocyte adhesion, among other inflammatory responses that contribute to the injury of the affected tissue [54,65,66].

Lipids in Cerebral Ischemia

When cerebral ischemia occurs, the flow of blood to the brain is interrupted by an obstruction, due to atherothrombotic or an embolism. The first is caused by the deposit and infiltration of lipids in the walls of arteries and the second occurs when a clot formed in another part of the body moves to the brain [67]. Intracellular levels of calcium strongly increase during cerebral ischemia acidosis and damage induced by free radicals [68]. This increase produces the activation of sphingomyelinasases and phospholipases A2, C and D that in turn favor other excitotoxic processes [69–71]. In addition, the inflammatory response after ischemia also alters lipid metabolism by increasing the production of eicosanoids, ceramides and free radicals promoting excitotoxicity and mitochondrial dysfunction [72–74].

Phospholipids in cerebral ischemia

Phospholipases constitute a group of enzymes that catalyze the hydrolysis of phospholipids and play a principal role in the maintenance and production of lipid mediators, which regulate cellular activity. The increase of these enzymes has been implicated in pathological conditions, including neuronal damage in ischemic response [74,75]. Phospholipases in the CNS are responsible of destabilization of the membrane through the degradation of phospholipids, increase of calcium influx [64], the release of Arachidonic acid (AA) and activation of the metabolism by cyclooxygenases/ lipoxygenases [77,78].

Cerebral ischemia is accompanied by the stimulation of isoforms of PLA2, massive release of free fatty acids, and increase in levels of LPC (Figure 5), which inhibits phosphocholine cytidylyltransferase (CTP); an enzyme that modulates PC synthesis [79]. Also, reduction of PC, PI, phosphatidylserine (PS) and cardiolipin after transient cerebral ischemia between other effects in lipid mediators have been described [77,80].
On the other hand, there are other investigations in neuroprotection that involve different chemical substances, for example, estradiol, statins, among other substances. Likewise, therapies that use preconditioning through hyperoxia or enriched environment have shown recovery of vital functions in the areas affected by ischemia. Natural products are being studied for the treatment of different CNS diseases such as CVD due to their antioxidant, anti-inflammatory properties, among others, which could be involved in various beneficial mechanisms against the progress of the disease.

Neuroprotection by natural products in cerebral ischemia

Currently, about 80% of the world population uses medicines that are derived directly or indirectly from plants. Natural products offer a wide variety of biological effects: anti-inflammatory, anticancer, antiviral, antithrombotic, antioxidant, anti-nociceptive, among others. In CNS diseases, some natural products have an anticonvulsant, analgesic, anxiolytic, antidepressant effect, in addition to improving memory and cognition when they are frequently administered. Thus, natural products are considered as a source of potential molecules in the field of neuroprotection.

Current therapy in Cerebral ischemia

With the aim of reducing the consequences of cerebral ischemia, numerous pharmacological agents have been used to evaluate their potential to prevent the destructive pathophysiology of stroke and protect the brain. Therapeutic approaches have been addressed to decrease the effects of excitatory amino acids such as glutamate, dampening calcium fluxes in the cell membrane, and regulating injury from inflammation, free radical damage, and intracellular enzymes. Early studies were unsuccessful by the late administration of therapy within the 4-6 hours therapeutic window for brain reperfusion. As example, thrombolytic therapies are addressed to restore perfusion in the tissue and minimize the damage. Currently, the only pharmacological therapy approved for clinical use in acute cerebral ischemia is the recombinant tissue plasminogen activator (rtPA), which converts the serine protease zymogen plasminogen into its active fibrin dissolving form plasmin. It is estimated that only 3% to 5% of stroke patients reach a hospital on time to be considered for thrombolytic agent; the same occurs with other medications such as intraarterial prourokinase.

Therapeutic properties of Linalool

Linalool (C10H18O), so-named 3,7-dimethyl-1,6-octadien-3-ol (Figure 6), is an acyclic monoterpene tertiary alcohol detected in essential oils of diverse plant species. Linalool has been reported over 200 monocotyledonous and dicotyledonous vegetal species extent across the world. Linalool is present mostly in plant families: Lamiaceae (genus Lavandula), Lauraceae (genus Cinnamomum) and Apiaceae (genus Coriandrum).

Pereira et al., 2018 described the characteristics of linalool such a molecule with a small molecular weight functionalized with a hydroxyl group. The alcohol functional group
Linalool has been used in the pharmaceutical and food industry for its antimicrobial, antioxidant and antifungal properties. Linalool exhibit a wide number of relevant bioactive properties, including anti-inflammatory, antioxidant, antinociceptive, anxiolytic, among others as we can observe in Table 1. These biological properties suggest that linalool could be a candidate compound for an effective therapy for improving cognitive function in neurodegenerative diseases.

### Table 1. Linalool bioactive properties and main underlying mechanisms of action

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<th>Mechanism of action</th>
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<td></td>
<td>Inhibition the production of inflammatory cytokines</td>
<td>[112]</td>
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<td></td>
<td>(TNF-α, IL-6, IL-1β, IL-8 &amp; MCP-1)</td>
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<td></td>
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### Thesis outline

Neurodegenerative diseases are hereditary and sporadic, characterized by progressive nervous system dysfunction. The majority of these diseases do not have a causal treatment, and we have worked in the understanding of the pathology to achieve this goal. My thesis was focused on evaluating the therapeutic use of the monoterpene linalool in AD and cerebral ischemia. Likewise, we used a lipidomic approach to understand the alterations
in these diseases using animal models and post-mortem tissues from patients with AD and CADASIL. Thus, we aimed to identify possible lipid biomarkers that reflect the progression of the disease and propose novel therapeutic targets against neurodegeneration.

Chapter 1 provides a brief review of the literature of the diseases that we will present in the other chapters. We use a lipidomic approach in AD and cerebral ischemia to better understand how lipids affect the pathology of these diseases. Also, we introduced the information about the standard treatments and promising natural products in neuroprotection.

In chapter 2, we took a look at the protective properties of linalool in a model of AD. For this purpose, we supply linalool for three months on aged triple transgenic model mice. We evaluated the reactivity of βA plaques, neurofibrillary tangles (NFT), astrocytes and microglia by immunostaining in different areas of the brain. Besides, we checked pro-inflammatory markers as p38 MAPK, NOS2, COX2, and IL-1β by western blot and ELISA. We also investigated the spatial memory and anxiolytic behavior in these animals by Morris water Maze and Elevated Plus Maze.

In chapter 3, we analyzed changes in the central and peripheral phospholipid profiles in ischemic rats and we determined if Linalool could modify them. We used an in vitro model of glutamate excitotoxicity where we studied LDH release, ATP levels and morphology of neurons and astrocytes from hippocampus and cortex. Besides, we used an in vivo global ischemia where we administered linalool orally for a month and we performed a behavioral test and lipidomic analysis using mass spectrometry. We evaluated astrocytes, microglia, and COX-2 by immunostaining in the hippocampus.

In chapter 4, we investigated the potential neuroprotective role of linalool on glutamate-induced mitochondrial oxidative stress in immortalized neuronal HT-22 cells. In addition, we also studied whether linalool is able to induce neuroprotection in organotypic hippocampal slices as ex vivo model for stroke, with NMDA as a stimulus for induction of excitotoxicity. We detected cell viability by real-time cell impedance measurements, MTT assay, and analysis of Annexin V/PI. We evaluated the morphology of mitochondria with MitoTracker and the production of ROS, calcium levels, and mitochondrial membrane potential by FACS. Besides, we use high-resolution respirometry, and Seahorse Extracellular flux analyze to observe the activity of linalool in the mitochondria complexes.

In chapter 5, we explored phospholipid profiles a month postischemia in cognitively impaired rats. We used a two-vessel occlusion (2-VO) model to generate brain ischemia, and we check alterations in myelin, endothelium, astrocytes, and inflammation mediators. Likewise, a lipidomic analysis was performed via mass spectrometry in the hippocampus and serum a month postischemia using univariate and multivariate statistical analysis.

In the same way, in chapter 6, we investigated the post-mortem temporal cortex grey matter, corpus callosum, and CSF, to define potential similarities and differences on the phospholipid profile that could to distinguish cognitively the healthy group from those with CADASIL and Sporadic AD (SAD). We used mass spectrometry, and lipid profile was subjected to multivariate analysis in order to discriminate between dementia groups and healthy controls.

In chapter 7, we present a review of the role of microglia in neurodegenerative diseases such as AD, PD, and we provide and update on the current model systems to study microglia, including cell lines, iPSC-derived microglia, and integration into 3D brain assembloids. Thus, we showed relevant strategies to research the role of microglia in neurodegeneration and we underlined platforms that could help to find efficient therapies. In this way, in chapter 8, we present the results of the differentiation protocol of human microglia using a modified protocol of Douvaras et al., 2017 [150] and the generation of organoids based on Lancaster et al., 2013 [151]. In this chapter we demonstrate the maturity of iPSC-derived microglia and its functionality through stimulation with LPS and alpha-synuclein and by phagocytosis assays.

Finally, in chapter 9, the results of the studies described in the thesis were discussed. Moreover, perspectives for future research and possible clinical implications of the research are addressed.
Chapter 1

General Introduction


Chapter 1

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