Unraveling molecular signalling 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]. 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 [4].

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 [5,6].

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 [10].

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 carotenoids [12,14].

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](image_url)
and familial and sporadic late-onset AD. 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.

**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. 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.

Publications from late 1980 and 1990 suggested that decreased and alterations in brain phospholipid metabolism could be connected with AD. Increase in the activity of PLA isoforms and lysospholipases elevation in phosphodiester, phosphononoesters, fatty acids, prostaglandins, isoprostanes, 4-hydroxynonenals, and other lipid mediators has been reported in AD.

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

A reduction of Phosphatidylinositol (PI) levels and phosphatydilethanolamine (PE) levels were found in post-mortem brain samples from individuals with AD compared to controls. In parallel, a decreased of phosphatydilcholine (PC) or unchanged PC levels have been reported. Also, levels of Lyso-phosphatydilcholine (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. 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).

**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.
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 hour 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 an intracellular 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 an intracellular acidification. In turn favor other excitotoxic processes

Figure 4. Illustration of the penumbra concept. Infarct core (red): infarcted tissue. Penumbra (orange): salvageable tissue at risk for infarction in case of persistence vessel occlusion. Oligemia (yellow): hypoperfused tissue without risk for infarction. Cerebral blood flow decreases in direction to the infarct core. Decreased blood flow can be compensated by an increased oxygen extraction fraction and vasodilation of collateral vessels sufficiently enough in the oligemia but not in the penumbra. Figure from Simon et al., 2017 [57].

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 [57]. Intracellular levels of calcium strongly increase during cerebral ischemia acidosis and damage induced by free radicals [58]. This increase produces the activation of sphingomyelinases and phospholipases A2, C and D that in turn favor other excitotoxic processes [59–62]. 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 [71–73].

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].

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 [80]. If these conditions are prolonged for a long time will produce a deficit in essential proteins that allow cell survival [84]. 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 [65,66].
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.

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...
present in the chemical structure of linalool confers polarity to the compound, making it chemically reactive. In terms of solubility, linalool is poorly soluble in water due to the hydrocarbon apolar structure. In contrast, linalool is highly soluble in organic solvents (alcohol, chloroform, ether, etc.), fixed oils and propylene glycol [108,109].

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.

**Microglia**

Microglia are parenchymal tissue macrophages with thin branching processes (“ramified,” or treelike) that represent 10% of cells in the CNS [139,140]. Microglia operate as brain macrophages but are different from other tissue macrophages owing to their homeostatic phenotype and regulation in the CNS microenvironment. Microglia are in charge of the phagocytosis of microbes, dead cells, damaged synapses, protein aggregates, and other particulate and soluble antigens that may threaten the CNS. Additionally, they are the first source of proinflammatory cytokines becoming crucial mediators of neuroinflammation and modulating a broad spectrum of cellular responses [141].

Microglia arise from the hemangioblastic mesoderm enabling them to proliferate and self-renew, however, representing a non-replenished population of mitotic cells, these functions are subject to a variety of age-dependent changes due to telomere shortening [142]. Most notably, age-related microglial atrophy indicates incidence of pathology due to reduced neuroprotection and enhanced neurodegeneration [143]. Microglia become less ramified and densely packed [142]. Most notably, age-related microglial atrophy indicates incidence of pathology due to reduced neuroprotection and enhanced neurodegeneration [143]. Microglia shade cytosolic accumulations of lipofuscin granules, decreased proteolytic activity, and increased release of pro-inflammatory markers (e.g. a constant state of activation) [143]. Release of cytokines as well as neurotoxic molecules may contribute to chronic brain inflammation and impaired blood-brain barrier integrity [144].

Dysfunctional microglia are associated with several pathologies of the brain such as Alzheimer’s disease where microglia cluster around β plaques seemingly incapable of phagocytosis and amyotrophic lateral sclerosis (ALS) where they are involved in the release of pro-inflammatory mediators, as we can observe in Figure 7 [135,146]. Moreover, their chronic activation had been linked to multiple sclerosis and Parkinson’s disease (PD), whereas impaired phagocytosis and pruning activities are associated with schizophrenia and autism spectrum disorders [147,148].

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

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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 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 distinguish cognitively 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.
References


Chapter 1

General Introduction

Chapter 1

General Introduction


