Unraveling molecular signaling in neurodegenerative diseases

Sabogal Guaqueta, Angelica

DOI:
10.33612/diss.111514738

Unraveling molecular signaling in neurodegenetive diseases:
Focus on a protective mechanism mediated by linalool

Angélica María Sabogal Guáqueta

Angélica María Sabogal Guáqueta

Unraveling molecular signaling in neurodegenetive diseases:
Focus on a protective mechanism mediated by linalool

ISBN: 978-94-034-2337-1 (e-book)
ISBN: 978-94-034-2336-4 (print)

Cover and layout design by Claudia González Arévalo
claudia@tangroop.com

Printed by IPSKAMP printing
https://www.ipskampprinting.nl

university of groningen

Unraveling molecular signaling in neurodegenerative diseases:
Focus on a protective mechanism mediated by linalool

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Monday 13 January 2020 at 16.15 hours

by

Angélica María Sabogal Guáqueta

born on 12 October 1987
in Ibagué, Colombia

Supervisors
Prof. H.W.G.M. Boddeke

Co-supervisor
Dr. A.M. Dolga
Prof. G.P. Cardona Gomez

Assessment Committee
Prof. M. Schmidt
Prof. U.L.M. Eisel
Prof. C. Culmsee

Paranymphs:
Pien Goldsteen
Asmaa Oun

Table of content

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

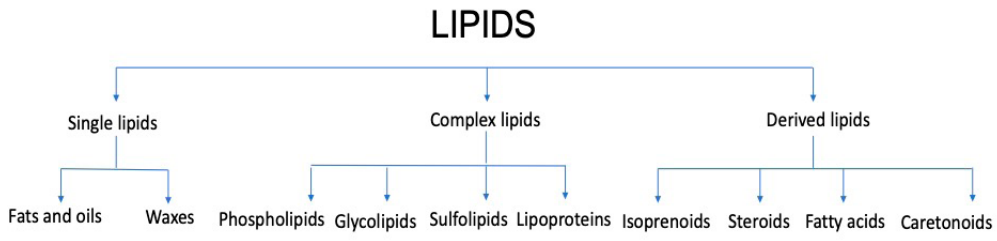


Figure 1. Classification of lipids. Modified from Bailwad et al., 2014 [14]

and familial and sporadic late-onset AD [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 [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 [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 [22].

Publications from late 1980 and 1990 suggested that decreased and alterations in brain phospholipid metabolism could be connected with AD [21]. Increase in the activity of PLA_2 isoforms and lysophospholipases elevation in phosphodiesters, phosphomonoesters, fatty acids, prostaglandins, isoprostanes, 4-hydroxynonenals, and other lipid mediators has been reported in AD [23].

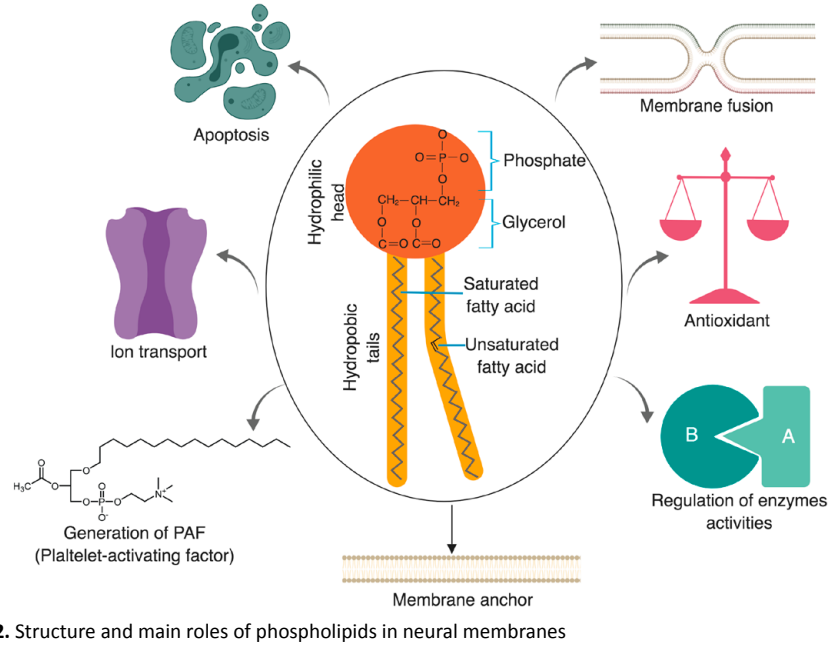


Figure 2. Structure and main roles of phospholipids in neural membranes

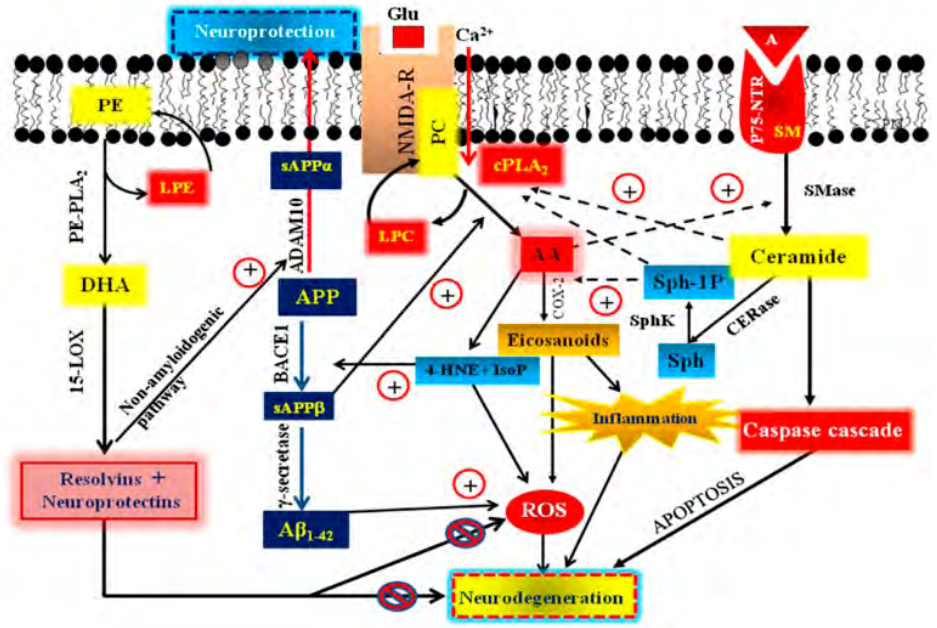


Figure 3. Principal molecular mechanism associated with lipid mediator-mediated neurodegeneration. AA, arachidonic acid; Aβ, β-amyloid; ADAM, A Disintegrin And Metalloprotease; sAPPα, soluble amyloid precursor protein α; sAPPβ, soluble amyloid precursor protein β; APP, amyloid precursor protein; BACE1, β-site APP cleavage enzyme; CERase, ceramidase; 4-HNE, 4-hydroxynonenals; IsoP, isoprostane; COX-2, cyclooxygenase 2; cPLA 2 , cytosolic phospholipase A 2; DHA, docosahexaenoic acid; 15-LOX, lipoxygenases; 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 [15].

A reduction of Phosphatidylinositol (PI) levels [24] and phosphatydilethanolamine (PE) levels were found in post-mortem brain samples from individuals with AD compared to controls [25,26]. In parallel, a decreased of phosphatydilcholine (PC) or unchanged PC levels [21,25] 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 [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) [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 [28].

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 [29] 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 [30]. However, adverse effects such as meningoencephalitis have been reported, resulting in discontinuation of the treatment [31]. 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 [32].

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 [33]. 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 [33–39]. 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 [40].

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 [33].

Cerebrovascular diseases

Cerebrovascular diseases (CVD) are the third cause of death in the world [41,42] and the second in Latin America after 45 years old according to the Pan American Health Organization [43]. 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 [44].

Cerebral strokes can be divided into ischemic and hemorrhagic, with an incidence of 84 and 16%, respectively [45]. 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 [46].

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

CADASIL is the most common monogenetic cause of adult-onset progressive cerebrovascular disease [47]. 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 [48,49].

CADASIL generally affects young people, and the first ischemic strokes occurs between 30 and 66 years of age [50]. 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 [51]. 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 [49,52].

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 increase of sodium and calcium ions [58,59]. 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 [60–62].

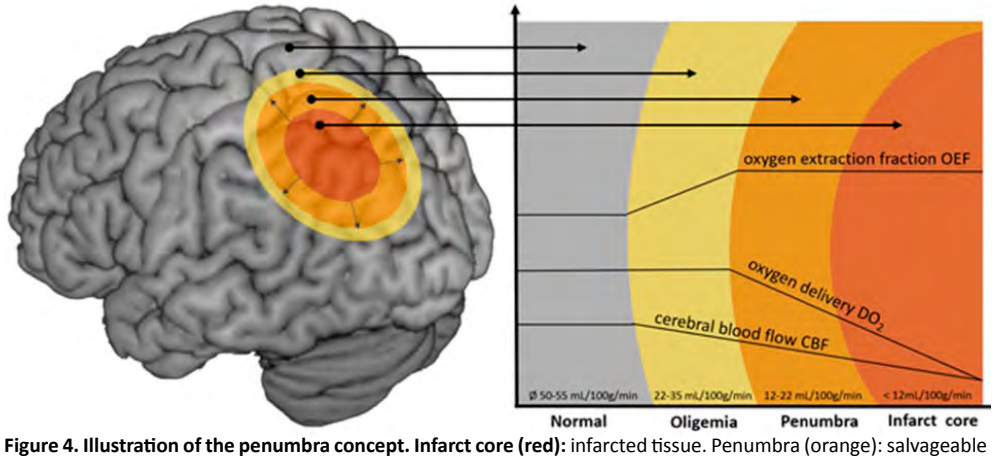


Figure 4. Illustration of the penumbra concept. Infarct core (red): infarcted tissue. Penumbra (orange): salvageable tissue at risk for infarction in case of persistence vessel occlusion. Oligemia (yellow): hypoperfused tissue without risk for infarction. Cerebral blood flow decreases in direction to 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]

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 [63]. 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 sphingomyelinases and phospholipases A2, C and D that in turn favor other excitotoxic processes [67,69,70]. 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 [66], 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 PLA_2, 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 [27,80]

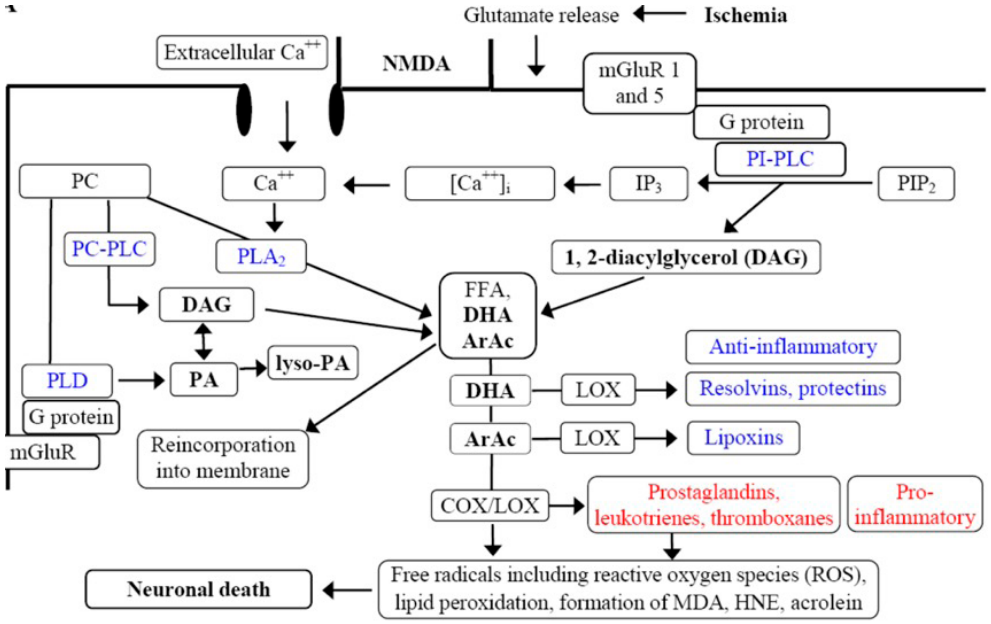


Figure 5. Lipid metabolism in ischemic neuronal death Activation of phospholipases (PLA_2, PC-PLC, PI-PLC, and PLD) following cerebral ischemia results in a release of lipid second messengers 1,2-diacylglycerol (DAG), phosphatidic acid (PA), lyso-phosphatidic acid (lyso-PA), docosahexaenoic acid (DHA), and arachidonic acid (ArAc). PA and DAG can be readily inter-converted by phosphohydrolases and DAG-kinases. ArAc undergoes further metabolism by cyclooxygenases/lipoxygenases (COX/LOX) to generate important signaling and vasoactive eicosanoids. Free radicals are formed during ArAc metabolism by COX/LOX and free radical generation can be induced by eicosanoids. ArAc generates pro-inflammatory prostaglandins, leukotrienes, and thromboxanes as well as LOX-generated anti-inflammatory lipoxins. Through the LOX pathway, DHA is metabolized to anti-inflammatory resolvins and protectins, including 10,17S-docosatriene (Neuroprotectin D1), an endogenous neuroprotectant. Figure from Adibhatla & Hatcher 2007 [80].

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 [81]. 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 [82,83]. It is estimated that only 3% to 5% of stroke patients reach a hospital on time to be considered for thrombolytic agent [84]; the same occurs with other medications such as intraarterial prourikinase [85].

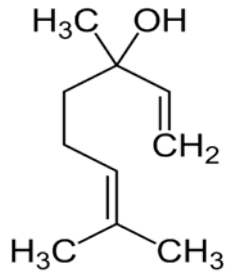


Figure 6. The structural formula of linalool

On the other hand, there are other investigations in neuroprotection that involve different chemical substances, for example, estradiol [86], statins [87], among other substances [88]. Likewise, therapies that use preconditioning through hyperoxia or enriched environment have shown recovery of vital functions in the areas affected by ischemia [89,90]. 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 [91].

Neuroprotection by natural products in cerebral ischemia

Currently, about 80% of the world population uses medicines that are derived directly or indirectly from plants [92,93]. Natural products offer a wide variety of biological effects: anti-inflammatory, anticancer, antiviral, antithrombotic, antioxidant, anti-nociceptive, among others [94,95]. In CNS diseases, some natural products have an anticonvulsant, analgesic, anxiolytic, antidepressant, antioxidant effect, in addition to improving memory and cognition when they are frequently administered [96–100]. Thus, natural products are considered as a source of potential molecules in the field of neuroprotection.

Natural products are reported in the treatment of different ischemia models. They can increase neurogenesis [101], decrease inflammatory responses [102], reduce cerebral edema [103], prevent breakdown of the blood-brain barrier [104], among other properties.

Therapeutic properties of Linalool

Linalool ($C_{10}H_{18}O$), 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 [105,106]. Linalool has been reported over 200 monocotyledonous and dicotyledonous vegetal species extent across the world [106]. Linalool is present mostly in plant families: Lamiaceae (genus Lavandula), Lauraceae (genus Cinnamomum) and Apiaceae (genus Coriandrum) [107,108].

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 to 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 [142]. Microglia become less ramified and dynamic, show 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 βA 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 [145,146]. 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

Bioactive property	Mechanism of action	References
Anti-inflammatory	Inhibition of COX-2	[110]
	Inhibition of the NF- κB pathway	[111,112]
	Inhibition the production of inflammatory cytokines (TNF-α, IL-6, IL-1β, IL-8 & MCP-1	[112]
	Activation of the Nrf2/HO-1 signaling pathway	[113,114]
	Inhibition of NO production	[115]
Antioxidant	Reduction of oxidative stress induced by H_2O_2	[116]
	Radicals scavenging activity	[117]
Anticancer & Antiproliferative	Cell cycle arrest	[118,119]
	Apoptosis induction	[120]
	Activation of immune cells (T helper cells)	[118]
	Prevention of the overexpression of angiogenic factors (VEGF and TGF-β1)	[121]
Antihyperlipidemic	Inhibition of lipid production	[122]
	HMGCR expression reduction	[123]
	Inhibition of proliferation and cholesterogenesis	[124]
	PPARα agonist & reduction of TGA in plasma que	[125]
Antinoceptive & Analgesic	Activation of peripheral opioid mechanisms	[126]
	Interaction with ionotropic glutamatergic receptors (NMDA)	[127]
Anxiolytic & Anti-depressant	Interaction with neuronal excitability through the inhibition of volt-age-dependent sodium channels	[128]
	Interaction with the monoaminergic system, (serotonin 1 A receptor and α2 adrenergic receptors)	[128–130]
	Alteration of the expression levels of genes associated with the synaptic transmission and MHC class I	[131]
Neuroprotective	Inhibition of the excitability of peripheral nervous system by interaction with protein membranes responsible for ATP generation, such as Na + channels	[132]
	Axonal regeneration	[133]
	Inhibition of presynaptic action potential propagation (linalool decreas-es smooth muscle GPCR)	[134]
	Regulation of glutamatergic system	[135,136]
	Neuroprotective agent against the neurotoxicity induced by acrylamide	[137]
	Cell death reduction in a glucose/serum deprivation model	[138]

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

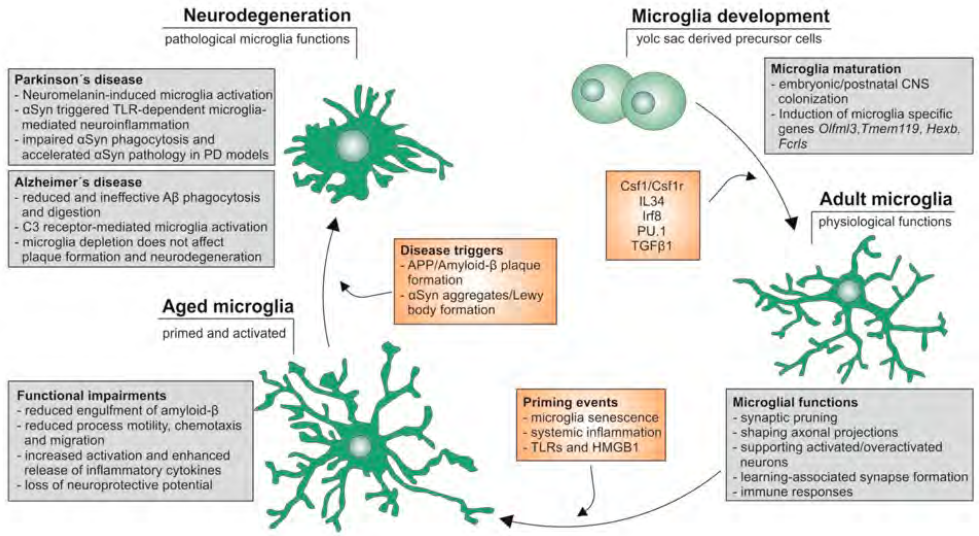


Figure 7. Microglia life cycle. Schematic summary of microglial development, maturation as well as aging and the functional changes influencing onset, severity and progression of neurodegeneration in Alzheimer's disease and Parkinson's disease. Figure from Spittau, 2017 [149].

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 excitotoxity. 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.

References

[1] A.D. Gitler, P. Dhillon, J. Shorter, Neurodegenerative disease: models, mechanisms, and a new hope, Dis. Model. Mech. 10 (2017) 499–502. doi:10.1242/dmm.030205.

[2] T. Wyss-coray, Ageing, neurodegeneration and brain rejuvenation, Nature. 539 (2016) 180–186. doi:10.1038/nature20411.

[3] B.N. Dugger, D.W. Dickson, Pathology of Neurodegenerative Diseases, Cold Spring Harb. Perspect. Biol. 9 (2017) 1–22. doi:10.1101/cshperspect.a028035.

[4] F. Coppede, M. Mancuso, G. Siciliano, Æ.L. Migliore, L. Murri, Genes and the Environment in Neurodegeneration, Biosci. Rep. 26 (2006) 341–367. doi:10.1007/s10540-006-9028-6.

[5] J.P. Taylor, J.P. Taylor, J. Hardy, K.H. Fischbeck, Toxic Proteins in Neurodegenerative Disease, Science (80-.). 296 (2002) 1991–1995. doi:10.1126/science.1067122.

[6] C.A. Lane, Alzheimer's disease, Eur. J. Neurol. 1 (2017) 59–70. doi:10.1111/ene.13439.

[7] M. Crous-bou, C. Minguillón, N. Gramunt, J.L. Molinuevo, Alzheimer's disease prevention: from risk factors to early intervention, Alzheimer´s Res. Ther. 9 (2017) 1–9. doi:10.1186/s13195-017-0297-z.

[8] H. Jahn, Memory loss in Alzheimer´s disease, Dialogues Clin. Neurosci. 15 (2013) 445–454.

[9] P. Tellechea, N. Pujol, B. Echeveste, J. Arbizu, M. Riverol, Early- and late-onset Alzheimer disease: Are they the same entity?, Neurología. 33 (2018) 244–253. doi:10.1016/j.nrl.2015.08.002.

[10] L.M. Ittner, J. Götz, Amyloid-β and tau — a toxic pas de deux in Alzheimer's disease, Nat. Rev. Neurosci. 12 (2011) 67–72. doi:10.1038/nrn2967.

[11] S.A. Frautschy, G.M. Cole, Why Pleiotropic Interventions are Needed for Alzheimer's Disease, Mol. Neurobiol. 41 (2010) 392–409. doi:10.1007/s12035-010-8137-1.

[12] E. Fahy, D. Cotter, M. Sud, S. Subramaniam, Lipid Classification, structures and tools, Biochim. Biophys. Acta. 1811 (2011) 637–647. doi:10.1016/j.bbalip.2011.06.009.

[13] K. Bozek, Y. Wei, Z. Yan, X. Liu, J. Xiong, M. Sugimoto, M. Tomita, S. Pääbo, C.C. Sherwood, P.R. Hof, J.J. Ely, Y. Li, D. Steinhauser, L. Willmitzer, P. Giavalisco, P. Khaitovich, Organization and Evolution of Brain Lipidome Revealed by Large-Scale Analysis of Human, Chimpanzee, Macaque, and Mouse Tissues, Neuron. 85 (2015) 695–702. doi:10.1016/j.neuron.2015.01.003.

[14] S. Bailwad, N. Singh, D. Jani, P. Patil, M. Singh, G. Deep, S. Singh, Alterations in Serum Lipid Profile Patterns in Oral Cancer: Correlation with Histological Grading and Tobacco Abuse, Oral Heal. Dent Manag. 13 (2014) 573–9. doi:10.4103/0973-1482.103517.

[15] V. Frisardi, F. Panza, D. Seripa, T. Farooqui, A.A. Farooqui, Glycerophospholipids and glycerophospholipid-derived lipid mediators : A complex meshwork in Alzheimer's disease pathology, Prog. Lipid Res. 50 (2011) 313–330. doi:10.1016/j.plipres.2011.06.001.

[16] A. Solomon, I. Kareholt, T. Ngandu, B. Wolozin, S. MacDonald, B. Winblad, A. Nissinen, J. Tuomilehto, H. Soininen, M. Kivipelto, Serum total cholesterol, statins and cognition in non-demented elderly, Neurobiol. Aging. 30 (2009) 1006–1009. doi:10.1016/j.neurobiolaging.2007.09.012.

[17] M.W. Wong, N. Braidy, A. Poljak, The application of lipidomics to biomarker research and pathomechanisms in Alzheimer's disease, Curr. Opin. Psychiatry. 30 (2017) 136–144. doi:10.1097/YCO.0000000000000303.

[18] G. Di Paolo, T. Kim, Linking Lipids to Alzheimer's Disease: Cholesterol and Beyond, Nat. Rev. Neurosci. 12 (2012) 284–296. doi:10.1038/nrn3012.Linking.

[19] A.M. Dupuy, E. Mas, K. Ritchie, B. Descomps, S. Badiou, J.P. Cristol, J. Touchon, The Relationship between Apolipoprotein E4 and Lipid Metabolism Is Impaired in Alzheimer's Disease, Gerontology. 47 (2001) 213–218.

[20] J.M. Castellano, J. Kim, F.R. Stewart, H. Jiang, R.B. Demattos, B.W. Patterson, A.M. Fagan, J.C. Morris, K.G. Mawuenyega, C. Cruchaga, A.M. Goate, K.R. Bales, S.M. Paul, R.J. Bateman, D.M. Holtzman, Human apoE Isoforms Differentially Regulate Brain Amyloid- b Peptide Clearance, Sci. Transl. Med. 3 (2011) 1–11. doi:10.1126/scitranslmed.3002156 Clearing.

[21] M. Kosicek, S. Hecimovic, Phospholipids and Alzheimer ' s Disease : Alterations , Mechanisms and Potential Biomarkers, Int. J. Mol. Sci. 14 (2013) 1310–1322. doi:10.3390/ijms14011310.

[22] Lodish, Biología celular y molecular, Médica Panamericana, 2005.

[23] A.A. Farooqui, W. Ong, L.A. Horrocks, Inhibitors of Brain Phospholipase A2 Activity: Their Neuropharmacological Effects and Therapeutic Importance for the Treatment of Neurologic Disorders, Pharmacol. Rev. 58 (2006) 591–620. doi:10.1124/pr.58.3.7.591.

[24] C.E. Stokes, J.N. Hawthorne, Reduced Phosphoinositide Concentrations in Anterior Temporal Cortex of Alzheimer-Diseased Brains, J. Neurochem. 48 (1987) 1018–1021.

[25] R.M. Nitsch, J.A.N.K. Blusztajn, A.G. Pittast, B.E. Slackt, J.H. Growdon, R.J. Wurtmant, Evidence for a membrane defect in Alzheimer disease brain, Proc. Natl. Acad. Sci. USA. 89 (1992) 1671–1675. doi:10.1073/pnas.89.5.1671.

[26] K. Wells, A.A. Farooqui, L. Liss, L.A. Horrocks, Neural Membrane Phospholipids in Alzheimer Disease, Neurochem. Res. 20 (1995) 1329–1333. doi:10.1007/BF00992508.

[27] A.A. Farooqui, Lipid Mediators and Their Metabolism in the Brain, Springer Science & Business Media., 2011.

[28] T.H. Ferreira-vieira, I.M. Guimaraes, F.R. Silva, F.M. Ribeiro, Alzheimer ' s Disease : Targeting the Cholinergic System, Curr. Neuropharmacol. 14 (2016) 101–115. doi :10.2174/1570159X13666150716165726.

[29] B. Lam, E. Hollingdrake, J.L. Kennedy, S.E. Black, M. Masellis, Cholinesterase inhibitors in Alzheimer's disease and Lewy body spectrum disorders: The emerging pharmacogenetic story, Hum. Genomics. 4 (2009) 91–106. doi:10.1186/1479-7364-4-2-91.

[30] A. Fettelschoss, F. Zabbel, M. Bachmann, Vaccination against Alzheimer disease An update on future strategies, Hum. Vaccin. Immunother. 10 (2014) 847–851. doi:10.4161/hv.28183.

[31] J. Delrie, P.J. Ousset, C. Caillaud, B. Vellas, Clinical trials in Alzheimer´s disease: immunotherapy approaches, J. Neurochem. 120 (2012) 186–193. doi:10.1111/j.1471-4159.2011.07458.x.

[32] G. Chen, P. Chen, H. Tan, D. Ma, F. Dou, J. Feng, Z. Yan, Regulation of the NMDA receptor-mediated synaptic response by acetylcholinesterase inhibitors and its impairment in an animal model of Alzheimer's disease, Neurobiol. Aging. 29 (2008) 1795–1804. doi:10.1016/j.neurobiolaging.2007.04.023.

[33] A.M. Sabogal-Guaqueta, E. Osorio, G.P. Cardona-Gómez, Flavonoids in Transgenic Alzheimer's Disease Mouse Models, in: Neuroprotective Eff. Phytochem. Neurol. Disord., John Wiley & Sons, 2017: pp. 43–63.

[34] V. Vingtdeux, U. Dreses-werringloer, H. Zhao, P. Davies, P. Marambaud, Therapeutic potential of resveratrol in Alzheimer's disease, BMC Neurosci. 9 (2008) 1–5. doi:10.1186/1471-2202-9-S2-S6.

[35] T. Akaishi, T. Morimoto, M. Shibao, S. Watanabe, K. Sakai-kato, Structural requirements for the flavonoid fisetin in inhibiting fibril formation of amyloid β protein, Neurosci. Lett. 444 (2008) 280–285. doi:10.1016/j.neulet.2008.08.052.

[36] L. Zhao, J. Wang, R. Liu, X. Li, J. Li, L. Zhang, Neuroprotective, Anti-Amyloidogenic and Neurotrophic Effects of Apigenin in an Alzheimer's Disease Mouse Model, Molecules. 18 (2013) 9949–9965. doi:10.3390/molecules18089949.

[37] J.M. Walker, D. Klakotskaia, D. Ajit, G.A. Weisman, W.G. Wood, S.B. Life, Beneficial Effects of Dietary EGCG and Voluntary Exercise on Behavior in an Alzheimer's Disease Mouse Model, J. Alzheimer's Dis. 44 (2015) 561–572. doi:10.3233/JAD-140981.

[38] A.S. DeToma, J. Choi, J.J. Braymer, M. Hee, Myricetin: A Naturally Occurring Regulator of Metal-Induced Amyloid-β Aggregation and Neurotoxicity, ChemBioChem. 12 (2011) 1198–1201. doi:10.1002/cbic.201000790.

[39] M. Venigalla, E. Gyengesi, G. Münch, Curcumin and Apigenin– novel and promising therapeutics against chronic neuroinflammation in Alzheimer's disease, Neural Regen. Res. 10 (2015) 1181–1185. doi:10.4103/1673-5374.162686.

[40] A.M. Sabogal-Guáqueta, J.I. Muñoz-Manco, J.R. Ramírez-Pineda, M. Lamprea-Rodriguez, E. Osorio, G.P. Cardona-Gómez, The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice, Neuropharmacology. 93 (2015) 134–145. doi:10.1016/j.neuropharm.2015.01.027.

[41] V.L. Feigin, B. Norrving, M.G. George, J.L. Foltz, G.A. Roth, Prevention of stroke: a strategic global imperative, Nat. Rev. Neurol. 12 (2016) 501–12. doi:10.1038/nrneurol.2016.107.

[42] D. Mozaffarian, E. Benjamin, A. Go, A. DK, M. Blaha, et al Cushman M, Heart disease and stroke statistics-2015 update: a report from the American Heart Association., Circulation. 131 (2015) e29-322. doi:10.1161/CIR.0000000000000152.

[43] PAHO, Causas principales de mortalidad en América Latina 2015, (2015). http://ais.paho.org/phip/viz/mort_causasprincipales_lt_oms.asp.

[44] P. Rodríguez-García, D. Rodríguez-García, Diagnosis of vascular cognitive impairment and its main categories, Neurologia. 30 (2015) 223–239. doi:10.1016/j.nrleng.2011.12.013.

[45] E. Díez-Tejedor, O. Del Brutto, J. Álvarez-Sabín, M. Muñoz, G. Abiusi, Clasificación de las enfermedades cerebrovasculares. Sociedad Iberoamericana de Enfermedades Cerebrovasculares, Neurología. 33 (2001) 455–464. doi:10.33588/rn.3305.2001246.

[46] N.S. Association, Understand Stroke, What Is Stroke? (2019). https://www.stroke.org/understand-stroke/what-is-stroke/hemorrhagic-stroke/.

[47] Y.R.Y. Tan, H.S. Markus, CADASIL: Migraine, Encephalopathy, Stroke and Their Inter-Relationships, PLOS ONE|. 11 (2016) 1–14. doi:10.1371/journal.pone.0157613.

[48] M. Gong, F. Rueschendorf, P. Marx, H. Schulz, H.-G. Kraft, N. Huebner, Koennecke, Hans-christian, Clinical and genetic features in a family with CADASIL and high lipoprotein (a) values, J Neurol. 257 (2010) 1240–1245. doi:10.1007/s00415-010-5496-5.

[49] H. Chabriat, A. Joutel, M. Dichgans, E. Tournier-lasserve, M. Bousser, Review CADASIL, Lancet Neurol. 8 (2009) 643–653. doi:10.1016/S1474-4422(09)70127-9.

[50] C. Opherk, N. Peters, J. Herzog, R. Luedtke, M. Dichgans, Long-term prognosis and causes of death in CADASIL: a retrospective study in 411 patients, Brain. 127 (2004) 2533–2539. doi:10.1093/brain/awh282.

[51] A. Bersano, G. Bedini, J. Oskam, C. Mariotti, F. Taroni, S. Baratta, E.A. Parati, CADASIL: Treatment and Management Options, Curr. Teatment Options Neurol. 19 (2017) 1–15. doi:10.1007/s11940-017-0468-z.

[52] S. Zhu, S.J. Nahas, CADASIL: Imaging Characteristics and Clinical Correlation, Curr. Pain Headache Rep. 20 (2016) 1–5. doi:10.1007/s11916-016-0584-6.

[53] J.F. Meschia, T. Brott, Ischaemic stroke, Eur. J. Neurol. 25 (2017) 35–40. doi:10.1111/ene.13409.

[54] M. Ahmad, S.H. Graham, Inflammation after stroke: Mechanisms and Therapeutic approaches, Transl. Stroke Res. 1 (2010) 74–84. doi:10.1007/s12975-010-0023-7.Inflammation.

[55] A.M. Kaufmann, A.D. Firlik, M.B. Fukui, L.R. Wechsler, C.A. Jungries, H. Yonas, Ischemic Core and Penumbra in Human Stroke, Stroke. 30 (1999) 93–99. doi:10.1161/01.STR.30.1.93.

[56] L. Wu, W. Wu, E.T. Tali, Oligemia, Penumbra, Infarction Understanding Hypoperfusion with Neuroimaging, Neuroimaging Clin. NA. 28 (2018) 599–609. doi:10.1016/j.nic.2018.06.013.

[57] J. Simon, W. Roland, G. Jan, M. Richard, M.H. P, L. David, Relevance of the cerebral collateral circulation in ischaemic stroke: time is brain , but collaterals set the pace, Swiss Med. Wkly. 147 (2017) 1–7. doi:10.4414/smw.2017.14538.

[58] T. Hayashi, K. Abe, Ischemic neuronal cell death and organellae damage, Neurol. Res. 26 (2004) 827–834. doi:10.1179/016164104X3770.

[59] A.M. Sabogal, C.A. Arango, G.P. Cardona, Á.E. Céspedes, Atorvastatin protects GABAergic and dopaminergic neurons in the nigrostriatal system in an experimental rat model of transient focal cerebral ischemia, Biomédica. 34 (2014) 207–217. doi:10.7705/biomedica.v34i2.1851.

[60] F. Fluri, M. Schuhmann, C. Kleinschnitz, Animal models of ischemic stroke and their application in clinical research, Drug Des. Devel. Ther. 9 (2015) 3445–3454. doi:10.2147/DDDT.S56071.

[61] L. Hertz, Neuropharmacology Bioenergetics of cerebral ischemia: A cellular perspective, Neuropharmacology. 55 (2008) 289–309. doi:10.1016/j.neuropharm.2008.05.023.

[62] J. Feber, S.T. Pavlidou, N. Erkamp, M.J.A.M. Van Putten, Progression of Neuronal Damage in an In Vitro Model of the Ischemic Penumbra, PLoS One. 11 (2016) 1–19. doi:10.5061/dryad.r6dv6.

[63] G. Turturici, G. Sconzo, F. Geraci, Hsp70 and Its Molecular Role in Nervous System Diseases, Biochem. Res. Int. (2011). doi:10.1155/2011/618127.

[64] X. Zhang, K. Deguchi, T. Yamashita, Y. Ohta, J. Shang, F. Tian, N. Liu, V.L. Panin, Y. Ikeda, T. Matsuura, K. Abe, Temporal and spatial differences of multiple protein expression in the ischemic penumbra after transient MCAO in rats, Brain Res. 1343 (2010) 143–152. doi:10.1016/j.brainres.2010.04.027.

[65] J. Huang, U.M. Upadhyay, R.J. Tamargo, Inflammation in stroke and focal cerebral ischemia, Surg. Neurol. 66 (2006) 232–245. doi:10.1016/j.surneu.2005.12.028.

[66] A. Ceulemans, T. Zgavc, R. Kooijman, S. Hachimi-idrissi, S. Sarre, Y. Michotte, The dual role of the neuroinflammatory response after ischemic stroke : modulatory effects of hypothermia, J. Neuroinflammation. 7 (2010) 74. doi:10.1186/1742-2094-7-74.

[67] P. Lipton, Ischemic Cell Death in Brain Neurons, Physiol. Rev. 79 (1999) 1431–1568. doi:10.1152/physrev.1999.79.4.1431.

[68] N. Khatri, H. Man, Synaptic activity and bioenergy homeostasis: implications in brain trauma and neurodegenerative diseases, Front. Neurol. 4 (2013) 1–11. doi:10.3389/fneur.2013.00199.

[69] J.W. Phillis, M.H. O'Regan, A potentially critical role of phospholipases in central nervous system ischemic, traumatic, and neurodegenerative disorders, Brain Res. Rev. 44 (2004) 13–47. doi:10.1016/j.brainresrev.2003.10.002.

[70] H. Tian, T. Qiu, J. Zhao, L. Li, J. Guo, Sphingomyelinase-induced ceramide production stimulate calcium-independent JNK and PP2A activation following cerebral ischemia., Brain Inj. 23 (2009) 1073–80. doi:10.3109/02699050903379388.

[71] J.G. Pilitsis, F.G. Diaz, M.H. O'Regan, J.W. Phillis, Differential effects of phospholipase inhibitors on free fatty acid efflux in rat cerebral cortex during ischemia-reperfusion injury, Brain Res. 951 (2002) 96–106. doi:10.1016/S0006-8993(02)03142-6.

[72] V. Capra, B. Magnus, S.S. Barbieri, M. Camera, E. Tremoli, G.E. Rovati, Eicosanoids and Their Drugs in Cardiovascular Diseases: Focus on Atherosclerosis and Stroke, Med. Res. Rev. 33 (2012) 364–438. doi:10.1002/med.21251.

[73] P.A. Fraser, The role of free radical generation in increasing cerebrovascular permeability, Free Radic. Biol. Med. 51 (2011) 967–977. doi:10.1016/j.freeradbiomed.2011.06.003.

[74] K. Arai, Y. Ikegaya, Y. Nakatani, I. Kudo, N. Nishiyama, N. Matsuki, Phospholipase A2 mediates ischemic injury in the hippocampus: a regional difference of neuronal vulnerability, Eur. J. Neurosci. 13 (2001) 2319–2323.

[75] T. Lin, Q. Wang, A. Simonyi, J. Chen, W. Cheung, Y.Y. He, J. Xu, A.Y. Sun, C.Y. Hsu, G.Y. Sun, Induction of secretory phospholipase A2 in reactive astrocytes in response to transient focal cerebral ischemia in the rat brain, J. Neurochem. 90 (2004) 637–645. doi:10.1111/j.1471-4159.2004.02540.x.

[76] Y. Inose, Y. Kato, K. Kitagawa, S. Uchiyama, N. Shibata, Activated microglia in ischemic stroke penumbra upregulate MCP-1 and CCR2 expression in response to lysophosphatidylcholine derived from adjacent neurons and astrocytes, Neuropathology. 35 (2015) 209–223. doi:10.1111/neup.12182.

[77] R.M. Adibhatla, J.F. Hatcher, R.J. Dempsey, Lipids and Lipidomics in Brain Injury and Diseases, 8 (2006) 314–321.

[78] A.M. Rao, J.F. Hatcher, R.J. Dempsey, Lipid Alterations in Transient Forebrain Ischemia: Possible New Mechanisms of CDP-Choline Neuroprotection, J. Neurochem. 75 (2000) 2528–2535.

[79] R.M. Adibhatla, J.F. Hatcher, R.J. Dempsey, Cytidine-5-Diphosphocholine Affects CTP- Phosphocholine Cytidylyltransferase and Lyso-Phosphatidylcholine After Transient Brain Ischemia, J. Neurosci. Res. 396 (2004) 390–396. doi:10.1002/jnr.20078.

[80] R.M. Adibhatla, Role of Lipids in Brain Injury and Diseases, Future Lipidol. 2 (2007) 403–422. doi:10.2217/17460875.2.4.403.

[81] T. Wieloch, K. Nikolich, Mechanisms of neural plasticity following brain injury, Curr. Opin. Neurobiol. 16 (2006) 258–264. doi:10.1016/j.conb.2006.05.011.

[82] A.J. Furlan, I.L. Katzan, L.R. Caplan, Thrombolytic Therapy in Acute Ischemic Stroke, Curr. Treat. Options Cardiovasc. Med. 5 (2003) 171–180. doi:10.1007/s11936-003-0001-4.

[83] K.R. Lees, E. Bluhmki, R. Von Kummer, T.G. Brott, D. Toni, J.C. Grotta, G.W. Albers, M. Kaste, J. Marler, S. Hamilton, B. Tilley, S. Davis, G. Donnan, W. Hacke, Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials, Lancet. 375 (2010) 1695–1703. doi:10.1016/S0140-6736(10)60491-6.

[84] J.M. Roth, Recombinant tissue plasminogen activator for the treatment of acute ischemic stroke, Proc (Bayl Univ Med Cent). 24 (2011) 257–259.

[85] G.J. Del Zoppo, Plasminogen activators and ischemic stroke: Conditions for acute delivery, Semin. Thromb. Hemost. 39 (2013) 406–425. doi:10.1055/s-0033-1338126.

[86] G.P. Cardona-Gómez, C. Arango-davila, J.C. Gallego-Gómez, A. Barrera-ocampo, H. Pimienta, L.M. Garcia-segura, Estrogen dissociates Tau and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor subunit in postischemic hippocampus, Neuroreport. 17

(2006) 1337–1341.

[87] J.A. Gutiérrez-vargas, A. Cespedes-rubio, G.P. Cardona-gómez, Perspective of synaptic protection after post-infarction treatment with statins, J. Transl. Med. 13 (2015) 1–9. doi:10.1186/s12967-015-0472-6.

[88] R.A.G. Patel, P.W. Mcmullen, Neuroprotection in the Treatment of Acute Ischemic Stroke, Prog. Cardiovasc. Dis. 59 (2017) 542–548. doi:10.1016/j.pcad.2017.04.005.

[89] M.R. Bigdeli, Neuroprotection Caused by Hyperoxia Preconditioning in Animal Stroke Models, Sci. World J. 11 (2011) 403–421. doi:10.1100/tsw.2011.23.

[90] R.F. Villa, F. Ferrari, A. Moretti, Post-stroke depression: Mechanisms and pharmacological treatment, Pharmacol. Ther. 184 (2018) 131–144. doi:10.1016/j.pharmthera.2017.11.005.

[91] M.K. Parvez, Natural or Plant Products for the Treatment of Neurological Disorders: Current Knowledge, Curr. Drug Metab. 19 (2018) 424–428. doi:10.2174/1389200218666170710190249.

[92] V.A. Bhattaram, U. Graefe, C. Kohlert, M. Veit, H. Derendorf, Pharmacokinetics and Bioavailability of Herbal Medicinal Products, Phytomedicine. 9 (2002) 1–33. doi:10.1078/1433-187X-00210.

[93] S.T. Toenjes, J.L. Gustafson, Atropisomerism in medicinal chemistry: challenges and opportunities, Future Med. Chem. 10 (2018) 409–422. doi:10.4155/fmc-2017-0152.

[94] P. Arulselvan, M.T. Fard, W.S. Tan, S. Gothai, S. Fakurazi, M.E. Norhaizan, S.S. Kumar, Role of Antioxidants and Natural Products in Inflammation, Oxid. Med. Cell. Longev. 2016 (2016) 1–15. doi:10.1155/2016/5276130.

[95] M. Hye, J. Kim, I.A. Khan, L.A. Walker, S.I. Khan, Nonsteroidal anti-in fl ammatory drug activated gene-1 (NAG-1) modulators from natural products as anti-cancer agents, Life Sci. 100 (2014) 75–84. doi:10.1016/j.lfs.2014.01.075.

[96] K. Rezai-zadeh, R.D. Shytle, Y. Bai, J. Tian, H. Hou, T. Mori, J. Zeng, D. Obregon, T. Town, J. Tan, Flavonoid-mediated presenilin-1 phosphorylation reduces Alzheimer's disease beta-amyloid production, J. Cell. Mol. Med. 13 (2009) 574–588. doi:10.1111/j.1582-4934.2008.00344.x.

[97] C. Spagnuolo, S. Moccia, G.L. Russo, Anti-inflammatory effects of flavonoids in neurodegenerative disorders, Eur. J. Med. Chem. 153 (2018) 105–115. doi:10.1016/j.ejmech.2017.09.001.

[98] N. Ansari, F. Khodagholi, Natural Products as Promising Drug Candidates for the Treatment of Alzheimer's Disease: Molecular Mechanism Aspect, Curr. Neuropharmacol. 11 (2013) 414–429. doi:10.2174/1570159X11311040005.

[99] M.E. Pedersen, B. Szewczyk, K. Stachowicz, J. Wieronska, J. Andersen, G.I. Stafford, J. Van Staden, A. Pilc, A.K. Jäger, Effects of South African traditional medicine in animal models for depression, J. Ethnopharmacol. 119 (2008) 542–548. doi:10.1016/j.jep.2008.08.030.

[100] N.G.M. Gomes, M.G. Campos, J.M.C. Órfão, C.A.F. Ribeiro, Plants with neurobiological activity as potential targets for drug discovery, Prog. Neuropsychopharmacol. Biol. Psychiatry. 33 (2009) 1372–1389. doi:10.1016/j.pnpbp.2009.07.033.

[101] Q. Cai, Y. Li, J. Mao, G. Pei, Neurogenesis-Promoting Natural Product α -Asarone Modulates Morphological Dynamics of Activated Microglia, Front. Cell. Neurosci. 10 (2016) 1–13. doi:10.3389/fncel.2016.00280.

[102] N. Latruffe, L. Bioperoxil, S. Gabriel, U. De Bourgogne, B. Gabriel, F.- Dijon, Natural Products and Inflammation, Molecules. 22 (2017) 15–17. doi:10.3390/molecules22010120.

[103] K. Lee, I. Jo, S.H. Park, K.S. Kim, J. Bae, J. Park, B. Lee, H. Choi, Y. Bu, Defatted Sesame Seed Extract Reduces Brain Oedema by Regulating Aquaporin 4 Expression in Acute Phase of Transient Focal Cerebral Ischaemia in Rat, Phyther. Res. 1527 (2012) 1521–1527. doi:10.1002/ptr.4599 Defatted.

[104] Y. Liu, G. Hui, Y. Hao, X. Jie, C. Wei, The protective role of Tongxinluo on blood – brain barrier after ischemia– reperfusion brain injury, J. Ethnopharmacol. 148 (2013) 632–639. doi:10.1016/j.jep.2013.05.018.

[105] R.A. Raguso, More lessons from linalool: Insights gained from a ubiquitous floral volatile, Curr. Opin. Plant Biol. 32 (2016) 31–36. doi:10.1016/j.pbi.2016.05.007.

[106] A.C. Aprotosoaie, M. Hancianu, I. Costache, A. Miron, Linalool: a review on a key odorant molecule with valuable biological properties, Flavour Fragance J. 29 (2014) 193–219. doi:10.1002/ffj.3197.

[107] L. Caputo, L.F. Souza, S. Alloisio, L. Cornara, V. De Feo, Coriandrum sativum and Lavandula angustifolia Essential Oils: Chemical Composition and Activity on Central Nervous System, Int. J. Mol. Sci. 17 (2016) 2–12. doi:10.3390/ijms17121999.

[108] I. Pereira, P. Severino, A.C. Santos, A.M. Silva, E.B. Souto, Colloids and Surfaces B: Biointerfaces Linalool bioactive properties and potential applicability in drug delivery systems, Colloids Surfaces B Biointerfaces. 171 (2018) 566–578. doi:10.1016/j.colsurfb.2018.08.001.

[109] T. Ilc, C. Parage, B. Boachon, N. Navrot, D. Werck-reichhart, Monoterpenol Oxidative Metabolism: Role in Plant Adaptation and Potential Applications, Front. Plant Sci. 7 (2016) 1–16. doi:10.3389/fpls.2016.00509.

[110] X.J. Li, Y.J. Yang, Y.S. Li, W.K. Zhang, H. Bin Tang, α-Pinene, linalool, and 1-octanol contribute to the topical anti-inflammatory and analgesic activities of frankincense by inhibiting COX-2, J. Ethnopharmacol. 179 (2016) 22–26. doi:10.1016/j.jep.2015.12.039.

[111] M. Huo, X. Cui, J. Xue, G. Chi, R. Gao, X. Deng, S. Guan, J. Wei, L.W. Soromou, H. Feng, others, Anti-inflammatory effects of linalool in RAW 264.7 macrophages and lipopolysaccharide-induced lung injury model, J. Surg. Res. 180 (2013) e47-e54.

[112] J. Ma, H. Xu, J. Wu, C. Qu, F. Sun, S. Xu, Linalool inhibits cigarette smoke-induced lung inflammation by inhibiting NF-κB activation, Int. Immunopharmacol. 29 (2015) 708–713. doi:10.1016/j.intimp.2015.09.005.

[113] Y. Li, O. Lv, F. Zhou, Q. Li, Z. Wu, Y. Zheng, Linalool Inhibits LPS-Induced Inflammation in BV2 Microglia Cells by Activating Nrf2, Neurochem. Res. 40 (2015) 1520–1525. doi:10.1007/s11064-015-1629-7.

[114] Q. Wu, L. Yu, J. Qiu, B. Shen, D. Wang, L.W. Soromou, H. Feng, Linalool attenuates lung inflammation induced by Pasteurella multocida via activating Nrf-2 signaling pathway, Int. Immunopharmacol. 21 (2014) 456–463. doi:10.1016/j.intimp.2014.05.030.

[115] A.T. Peana, S. Marzocco, A. Popolo, A. Pinto, (-)-Linalool inhibits in vitro NO formation: Probable involvement in the antinociceptive activity of this monoterpene compound, Life Sci. 78 (2006) 719–723. doi:10.1016/j.lfs.2005.05.065.

[116] S. Celik, A. Ozkaya, Effects of intraperitoneally administered lipoic acid, vitamin E, and linalool on the level of total lipid and fatty acids in guinea pig brain with oxidative stress induced by H2O2., J. Biochem. Mol. Biol. 35 (2002) 547–52.

[117] H. Park, G.H. Seol, S. Ryu, I.-Y. Choi, Neuroprotective effects of (-)-linalool against oxygen-glucose deprivation-induced neuronal injury, Arch. Pharm. Res. 39 (2016) 555–564.

[118] M. Chang, Y. Shen, Linalool Exhibits Cytotoxic Effects by Activating Antitumor Immunity, Molecules. 19 (2014) 6694–6706. doi:10.3390/molecules19056694.

[119] X.-B. Sun, S.-M. Wang, T. Li, Y. Yang, Anticancer Activity of Linalool Terpenoid: Apoptosis Induction and Cell Cycle Arrest in Prostate Cancer Cells, Trop. J. Pharm. Res. 14 (2015) 619–625. doi:10.4314/tjpr.v14i4.9.

[120] T. Cerchiara, S. Straface, E. Brunelli, S. Tripepi, M.C. Gallucci, G. Chidichimo, Antiproliferative Effect of Linalool on RPMI 7932 Human Melanoma Cell Line: Ultrastructural Studies, Nat. Prod. Commun. 10 (2015) 1–3. doi:10.1177/1934578X1501000401.

[121] S. Gunaseelan, A. Balupillai, K. Govindasamy, K. Ramasamy, G. Muthusamy, M. Shanmugam, R. Thangaiyan, B.M. Robert, R.P. Nagarajan, V.K. Ponniresan, P. Rathinaraj, Linalool prevents oxidative stress activated protein kinases in single UVB-exposed human skin cells, PLoS One. 12 (2017) 1–20. doi:10.1371/journal.pone.0176699.

[122] B. Cheng, L. Sheen, S. Chang, Hypolipidemic effects of S-(+)-linalool and essential oil from Cinnamomum osmophloeum ct . linalool leaves in mice, J. Tradit. Chinese Med. Sci. 8 (2018) 46–52. doi:10.1016/j.jtcme.2017.02.002.

[123] S.Y. Cho, H.J. Jun, J.H. Lee, Y. Jia, K.H. Kim, S.J. Lee, Linalool reduces the expression of 3-hydroxy-3-methylglutaryl CoA reductase via sterol regulatory element binding protein-2- and ubiquitin-dependent mechanisms, FEBS Lett. 585 (2011) 3289–3296. doi:10.1016/j.febslet.2011.09.012.

[124] B. Rodenak Kladniew, M. Polo, S. Montero Villegas, M. Galle, R. Crespo, M. García De Bravo, Synergistic antiproliferative and anticholesterogenic effects of linalool, 1,8-cineole, and simvastatin on human cell lines, Chem. Biol. Interact. 214 (2014) 57–68. doi:10.1016/j.cbi.2014.02.013.

[125] H. Jun, J.H. Lee, J. Kim, Y. Jia, K.H. Kim, K.Y. Hwang, E.J. Yun, K.R. Do, S. Lee, Linalool is a PPAR α ligand that reduces plasma TG levels and rewires the hepatic transcriptome and plasma metabolome, 55 (2014). doi:10.1194/jlr.M045807.

[126] F.N. Souto-maior, D. Vilar, P. Regina, R. Salgado, L.D.O. Monte, D.P. De Sousa, R.N. De Almeida, R. Salgado, L.D.O. Monte, D.P. De Sousa, R. Nóbrega, N. Souto-maior, D. Vilar, P. Regina, R. Salgado, L.D.O. Monte, Antinociceptive and anticonvulsant effects of the monoterpene linalool oxide, 0209 (2017). doi:10.1080/13880209.2016.1228682.

[127] P.A. Batista, M.F. De Paula Werner, E.C. Oliveira, L. Burgos, P. Pereira, L.F. Da Silva Brum, G.M. Story, A.R.S. Santos, The antinociceptive effect of (-)-linalool in models of chronic inflammatory and neuropathic hypersensitivity in mice, J. Pain. 11 (2010) 1222–1229. doi:10.1016/j.jpain.2010.02.022.

[128] E. dos Santos, C. Maia, E. Fontes, A. Melo, B.G. Pinheiro, J.G.S. Maia, Linalool-rich essential oils from the Amazon display antidepressant-type effect in rodents, J. Ethnopharmacol. 212 (2018) 43–49. doi:10.1016/j.jep.2017.10.013.

[129] E. de Almeida, K. Rayane, D.O. Rafael, G. Bosco, L. Couto, A. Beatriz, M. Ishigami, Anxiolytic and Anticonvulsant Effects on Mice of Flavonoids, Linalool, and α -Tocopherol Presents in the Extract of Leaves of Cissus sicyoides L. (Vitaceae), J. Biomed. Biotechnol. (2009) 1–7. doi:10.1155/2009/274740.

[130] S.L. Guzmán-Gutiérrez, H. Bonilla-Jaime, R. Gómez-Cansino, R. Reyes-Chilpa, Linalool and β-pinene exert their antidepressant-like activity through the monoaminergic pathway, Life Sci. 128 (2015) 24–29. doi:10.1016/j.lfs.2015.02.021.

[131] K. Yoshida, N. Yamamoto, S. Fujiwara, A. Kamei, K. Abe, A. Nakamura, Inhalation of a racemic mixture (R,S) -linalool by rats experiencing restraint stress alters neuropeptide and MHC class I gene expression in the hypothalamus, Neurosci. Lett. 653 (2017) 314–319. doi:10.1016/j.neulet.2017.05.046.

[132] A.M. Venancio, F.W. Ferreira-da-silva, K.S. Silva-alves, H.D.C. Pimentel, M.M. Lima, M.F. De Santana, P.B. Alves, G. Batista, J.H. Leal-cardoso, M. Marchioro, Essential Oil of Ocimum basilicum L. and (−)-Linalool Blocks the Excitability of Rat Sciatic Nerve, Evidence-Based Complement. Altern. Med. 9012605 (2016) 1–7. doi:10.1155/2016/9012605.

[133] G. Kaka, K. Yaghoobi, S. Davoodi, S.R. Hosseini, Assessment of the Neuroprotective Effects of Lavandula angustifolia Extract on the Contusive Model of Spinal Cord Injury in Wistar Rats, Front. Neurosci. 10 (2016) 1–9. doi:10.3389/fnins.2016.00025.

[134] C. Poyton, M. Manchadi, M. Cheesman, N. Lavidis, Effects of Lavender and Linalool on Neurotransmission and Contraction of Smooth Muscle, Pharmacogn. Commun. 5 (2015) 217–225. doi:10.5530/pc.2015.3.7.

[135] L.F.S. Brum, T. Emanuelli, D.O. Souza, E. Elisabetsky, Effects of linalool on glutamate release and uptake in mouse cortical synaptosomes, Neurochem. Res. 26 (2001) 191–194.

[136] E. Elisabetsky, J. Marschner, D. Onofre Souza, Effects of linalool on glutamatergic system in the rat cerebral cortex, Neurochem. Res. 20 (1995) 461–465. doi:10.1007/BF00973103.

[137] S. Mehri, M.A. Meshki, H. Hosseinzadeh, Linalool as a neuroprotective agent against acrylamide-induced neurotoxicity in Wistar rats., Drug Chem. Toxicol. 38 (2015) 162–166. doi:10.3109/01480545.2014.919585.

[138] B. Alinejad, A. Ghorbani, H.R. Sadeghnia, Effects of combinations of curcumin, linalool, rutin, safranal, and thymoquinone on glucose/ serum deprivation-induced cell death, 3 (2013) 321–328. doi:10.22038/AJP.2013.418.

[139] M.L. Bennett, F.C. Bennett, S.A. Liddelow, B. Ajami, J.L. Zamanian, N.B. Fernhoff, S.B. Mulinyawe, C.J. Bohlen, A. Adil, A. Tucker, I.L. Weissman, E.F. Chang, G. Li, G.A. Grant, M.G. Hayden Gephart, B.A. Barres, New tools for studying microglia in the mouse and human CNS., Proc. Natl. Acad. Sci. U. S. A. 113 (2016) E1738–E1746. doi:10.1073/pnas.1525528113.

[140] A. Aguzzi, B.A. Barres, M.L. Bennett, Microglia: Scapegoat, Saboteur, or Something Else ?, Science (80-.). 339 (2013) 156–162. doi:10.1126/science.1227901.

[141] M. Colonna, O. Butovsky, Microglia Function in the Central Nervous System During Health and Neurodegeneration, Annu. Rev. Immunol. 35 (2017) 441–68. doi:10.1146/annurev-immunol-051116-052358.

[142] W.J. Streit, K.R. Miller, K.O. Lopes, E. Njie, Microglial degeneration in the aging brain– bad news for neurons?, Front. Biosci. 13 (2008) 3423–3438. doi:10.2741/2937.

[143] M.R. Damani, L. Zhao, A.M. Fontainhas, J. Amaral, R.N. Fariss, W.T. Wong, Age-related alterations in the dynamic behavior of microglia, Aging Cell. 10 (2011) 263–276. doi:10.1111/j.1474-9726.2010.00660.x.

[144] N.M. Luheshi, K.J. Kovács, G. Lopez-Castejon, D. Brough, A. Denes, Interleukin-1α expression precedes IL-1β after ischemic brain injury and is localised to areas of focal neuronal loss and penumbral tissues, J. Neuroinflammation. 8 (2011) 186. doi:10.1186/1742-2094-8-186.

[145] M.C. Geloso, V. Corvino, E. Marchese, A. Serrano, F. Michetti, N. D Ambrosi, The dual role of microglia in ALS: mechanisms and therapeutic approaches, Front. Aging Neurosci. 9 (2017) 242. doi:10.3389/fnagi.2017.00242.

[146] V. Navarro, E. Sanchez-Mejias, S. Jimenez, C. Muñoz-Castro, R. Sanchez-Varo, J.C. Davila, M. Vizuete, A. Gutierrez, J. Vitorica, Microglia in Alzheimer's disease: Activated, dysfunctional or degenerative, Front. Aging Neurosci. 10 (2018) 1–8. doi:10.3389/fnagi.2018.00140.

[147] P. Douvaras, B. Sun, M. Wang, I. Kruglikov, G. Lallos, M. Zimmer, C. Terrenoire, B. Zhang, S. Gandy, E. Schadt, D.O. Freytes, S. Noggle, V. Fossati, Directed Differentiation of Human Pluripotent Stem Cells to Microglia, Stem Cell Reports. 8 (2017) 1516–1524. doi:10.1016/j.stemcr.2017.04.023.

[148] J. Muffat, Y. Li, B. Yuan, M. Mitalipova, A. Omer, S. Corcoran, G. Bakiasi, L.-H. Tsai, P. Aubourg, R.M. Ransohoff, others, Efficient derivation of microglia-like cells from human pluripotent stem cells, Nat. Med. 22 (2016) 1358.

[149] B. Spittau, Aging microglia-phenotypes, functions and implications for age-related neurodegenerative diseases, Front. Aging Neurosci. 9 (2017) 1–9. doi:10.3389/fnagi.2017.00194.

[150] P. Douvaras, B. Sun, M. Wang, I. Kruglikov, G. Lallos, M. Zimmer, C. Terrenoire, B. Zhang, S. Gandy, E. Schadt, others, Directed differentiation of human pluripotent stem cells to microglia, Stem Cell Reports. 8 (2017) 1516–1524.

[151] M.A. Lancaster, M. Renner, C.-A. Martin, D. Wenzel, L.S. Bicknell, M.E. Hurles, T. Homfray, J.M. Penninger, A.P. Jackson, J.A. Knoblich, Cerebral organoids model human brain development and microcephaly., Nature. 501 (2013) 373–379. doi:10.1038/nature12517.

CHAPTER 2

Linalool reverses neuropathological and behavioral impairments in old triple transgenic Alzheimer's mice

Angélica María Sabogal Guáqueta[a], Edison Osorio[b], Gloria Patricia Cardona-Gómez[a]*

[a]Neuroscience Group of Antioquia, Cellular and Molecular Neurobiology Area - School of Medicine, SIU, University of Antioquia UdeA, Calle 70 No, 52-21, Medellin, Colombia

[b]Grupo de Investigación en Sustancias Bioactivas, Facultad de Ciencias Farmacéuticas y Alimentarias, Universidad de Antioquía UdeA, Calle 70 No, 52-21,Medellin, Colombia

Neuropharmacology 102 (2016) 111-120. doi: 10.1016/j.neuropharm.2015.11.002

Abstract

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disorder. Several types of treatments have been tested to block or delay the onset of the disease, but none have been completely successful. Diet, lifestyle and natural products are currently the main scientific focuses. Here, we evaluate the effects of oral administration of the monoterpene linalool (25 mg/kg), every 48 h for 3 months, on aged (21e24 months old) mice with a triple transgenic model of AD (3xTg-AD) mice. Linalool-treated 3xTg-AD mice showed improved learning and spatial memory and greater risk assessment behavior during the elevated plus maze. Hippocampi and amygdalae from linalool-treated 3xTg-AD mice exhibited a significant reduction in extracellular b-amyloidosis, tauopathy, astrogliosis and microgliosis as well as a significant reduction in the levels of the pro-inflammatory markers p38 MAPK, NOS2, COX2 and IL-1β. Together, our findings suggest that linalool reverses the histopathological hallmarks of AD and restores cognitive and emotional functions via an anti-inflammatory effect. Thus, linalool may be an AD prevention candidate for preclinical studies.

Keywords: Alzheimer's disease, linalool, behavior function, inflammation

1. Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disorder, is the most common form of dementia. AD is responsible for a considerable human, social and economic burden around the world (Association, 2014). In Latin America, the main causes of dementia are a sedentary lifestyle, metabolic disorder, and cardiovascular and cerebrovascular diseases (Kalaria et al., 2008). In general, AD patients present with a gradual deterioration of episodic memory, a global decline in cognitive function, and behavioral changes. AD symptoms are the clinical manifestations of a progressive accumulation of intra- and extracellular b-amyloid, the formation of neurofibrillary tangles (NFTs) and the extensive oxidative stress associated with neuron and synapse loss (Ittner and Gotz, 2011; Reitz and Mayeux, 2014).

The current standard pharmacotherapy for cognitive improvement in AD patients includes acetylcholinesterase inhibitors, such as galantamine, and the N-methyl-D-aspartate (NMDA) antagonist memantine, which promote cognitive function in patients with moderate to severe AD (Kang et al., 2014). However, the approval of these drugs has not been based on their ability to slow disease progression but on their ability to improve clinical symptomatology. Hence, only symptomatic drugs with transient benefits have been approved for clinical use in AD patients by the US Food and Drug Administration (FDA) (Bassil and Grossberg, 2009). The use of alternative therapies for neuroprotection is increasing; these alternatives include natural products, such as monoterpenes (Dinda et al., 2009; Tabassum et al., 2015).

(-) Linalool, one enantiomer of the naturally occurring monoterpene, is a major volatile component of essential oils in several aromatic plant species, such as *Lavandula angustifolia* Mill., *Melissa officinalis* L., *Rosmarinus officinalis* L. and *Cymbopogon citratus* DC. Interestingly, many linalool-producing species are traditionally used in folk medicine and in aromatherapy to relieve symptoms and to cure a variety of acute and chronic ailments (Batista et al., 2010; Elisabetsky et al., 1995). Linalool is widely used in the manufacture of fragrances for shampoos, soaps and detergents and in pharmaceutical drug formulations (Letizia et al., 2003; Mitic Culafic et al., 2009).

Linalool exhibits a variety of pharmacological effects, including antimicrobial, antileishmanial, anti-inflammatory, anti-oxidant and cardiovascular effects, in normotensive and hypertensive rats (Anjos et al., 2013; Beier et al., 2014; Celik and Ozkaya, 2002; Huo et al., 2013; Wu et al., 2014). The strong antioxidant activity of linalool inhibits LDL oxidation; this inhibition enhances cholesterol uptake via macrophage scavenger receptors. Linalool significantly reduced plasma TG, total cholesterol and HMG-CoA levels, demonstrating *in vivo* anti-atherogenic activity (Cho et al., 2011; Chung et al., 2008). Linalool has remarkable effects on the central nervous system (CNS), acting as a sedative, antinociceptive, anticonvulsant and anxiolytic (Batista et al., 2010; de Almeida et al., 2009; Elisabetsky et al., 1999; Linck

et al., 2009). Linalool also modulates glutamatergic neurotransmission both *in vitro* and *in vivo*, possibly through NMDA receptor interactions (Elisabetsky et al., 1995; Silva Brum et al., 2001). However, nothing is known about linalool's effect on AD neuropathology and behavioral impairments, which is the goal of the present study.

2. Materials and methods

2.1. Animals

Homozygous triple transgenic AD model (3xTg-AD) and non-transgenic (Non-Tg) mice (Oddo et al., 2003) from the in-house colony at the University of Antioquia maintained in the SIU (Sede de Investigación Universitaria) specific pathogen-free vivarium in Medellín, Colombia were used at ages from 18 to 21 months to obtain a homogenous penetrance of tauopathy. The mice were maintained on a 12:12 h dark: light cycle and received food and water ad libitum. The animals were handled according to Colombian standards (law 84/1989 and resolution 8430/1993) and guidelines. Special care was taken to minimize animal suffering and to reduce the number of animals used.

2.2. Administration of drugs

Linalool (Sigma Aldrich, Cat: L2602) was dissolved in phosphate-buffered saline (PBS). The 3xTg-AD mice received 25 mg/kg linalool or saline solution (vehicle) orally every 48 h for 3 consecutive months beginning at 18-21 months of age and were sacrificed at ages of 21-24 months (Fig. 1). The linalool dose (25 mg/kg) is not toxic (Brickers et al., 2003; Api et al., 2015), and the interval between the final drug treatment and the assays were selected based on previous *in vivo* studies (Huo et al., 2013; Mehri et al., 2014; Nascimento et al., 2014). We carefully monitored the general health of the mice throughout the linalool treatment and did not observe any adverse effects.

2.3. Histology

Twenty-four hours after the final behavioral test, the animals were anesthetized intraperitoneally using a mixture of ketamine (50 mg/kg) plus xylazine (20 mg/kg) and were perfused with normal saline and 4% paraformaldehyde (0.1 M PBS, pH 7.4). Brains were removed and post-fixed with 4% paraformaldehyde at 4˚C for 48 h and then cryopreserved with 30% sucrose and stored at -20˚C. The brains were sectioned (50 µm) with a Leica VT1000S vibrating blade microtome (Leica Microsystems, Germany).

2.4. Immunohistochemistry

The coronal sections (50 µm) were permeabilized and blocked with 0.3% Triton X-100 and 1% BSA in PBS and were then probed with primary antibodies: anti-beta amyloid antibody (beta amyloid 1-16 (6E10) Monoclonal #SIG-39320, Covance, 1:500), antiphospho-PHF-tau (pSer202/Thr205 Antibody (AT8) #MN1020, Thermo Scientific, 1:500), anti-GFAP

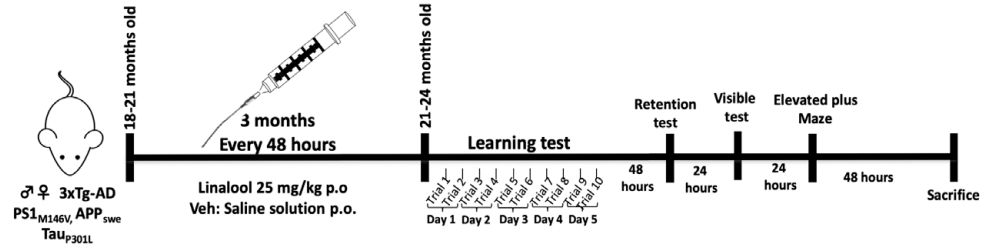


Fig. 1. Experimental design. Linalool (25 mg/kg) or saline were administered orally by gavage to 18-21-month-old Non-Tg and 3xTg-AD mice for 3 months, every 48 h. Learning and memory were evaluated in the Morris water maze test (five days, ten trials) at 21-24 months of age. Afterward, the elevated plus maze was performed over two days. Then, the mice were sacrificed for histological and biochemical analyses.

(Monoclonal Anti-Glial Fibrillary Acidic Protein, #G 3893, Sigma, 1:500), anti-Iba1 (Rabbit Anti-Iba1 (Ionized calcium binding adaptor molecule) #019e19741, Wako, 1:500) and the appropriate secondary antibodies (1:250 concentration, goat anti-rabbit IgG (H+L) biotin conjugated, Pierce #31822 or goat Anti-Mouse IgG (H+L) Biotin Conjugated Pierce #31800). Later, tissues were incubated with avidin biotin complex (ABC Standard Peroxidase Staining Kit, Pierce #32020, 1:250 reagent A:B) for 1h. Once the complex was removed, diaminobenzidine (DAB) was used as developer. The sections were dehydrated with alcohol, cleared with xylene and sealed with Consul-mount. The immunoreactivity in the tested areas was quantified at 10x or 40x magnification and analyzed using ImageJ 1.45 software (NIH, USA). The absence of primary antibody did not result in immunoreactivity. The CA1 and subiculum (hippocampus), entorhinal cortex (EC) and amygdala were evaluated at bregma 1.76 mm posterior to bregma (Paxinos and Franklin, 2004).

2.5. Immunofluorescence

Sections at the level of the bregma were rinsed in 0.1 M PBS and incubated for 10 min with 50 mM ammonium chloride to prevent autofluorescence. The sections were preincubated for 60 min at room temperature with Triton X-100 in PBS (TXPBS) and 3% BSA and were then incubated overnight at 4˚C with the following primary antibodies: anti-beta amyloid antibody (beta amyloid 1-16 (6E10) Monoclonal #SIG-39320, Covance, 1:500) and anti-Iba1 (Rabbit Anti-Iba1 (Ionized calcium binding adaptor molecule) #019-19741,Wako, 1:500). The primary antibodies were diluted in TxPBS and 1% BSA. Then, the sections were washed four times in 0.1 M PBS and incubated for 90 min at room temperature with mouse Alexa Fluor 488 or rabbit Alexa Fluor 594 secondary antibodies (1:1000; Molecular Probes, Eugene, OR). The sections were stained for 15 min at room temperature with Hoechst (1:1000; Sigma), washed four times in buffer, mounted on slides, and coverslipped with Gel Mount (Biomeda, Foster City, CA). The sections were observed and photographed with a motorized spinning disk confocal microscope (DSU; Olympus IX 81). Omission of the primary antibodies resulted in no staining.

2.6. Morris water maze test

Forty-eight hours after the final treatment, the animals were evaluated in the Morris water maze (MWM). A white plastic tank 1 m in diameter and 30 cm in height was filled with water (22 ± 2˚C) to a depth of 20 cm. The platform (7 cm diameter) was 1.5 cm below the surface of the water during spatial learning and 1.5 cm above the surface of the water during the visible session. Extra-maze visual cues around the room remained in a fixed position throughout the experiment. Ten sessions or trials were performed, two complete sessions per day over five days. "Each session consisted of four successive subtrials (30 s inter-trial interval), and each subtrial began with the mouse placed pseudo-randomly in one of four starting locations. The animals had been trained to stay on the platform for 30 s prior to the initial trial. The latency to reach the platform was evaluated using a visible platform to control for any difference in visual-motor abilities or motivation between the experimental groups. If a mouse did not locate the platform after a maximum of 60 s, it was gently guided to the platform. Then, the animals were then provided with 48 h of retention time, followed by a probe trial of spatial reference memory, in which the animals were placed in the tank without the platform for 60 s (Fig. 1). The latency to reach the exact former platform location and the number of crossings of the platform target quadrant were recorded during the probe trial. An automated system (Viewpoint, Lyon, France) recorded the behavior of the animals" (Sabogal-Guaqueta et al., 2015).

2.7. Elevated plus maze

Twenty-four hours after the visible test in the Morris water maze, the animals were exposed to the elevated plus maze (EPM). The apparatus was made of white Plexiglas and illuminated using approximately 30-40 lux. The EPM consisted of two open arms (30x 5x 0.25 cm) and two closed arms (30x 5x15 cm) extending from a common central platform (5x5 cm), and the entire apparatus was elevated by a single central support to a height of 60 cm above floor level. Each mouse was placed in the middle section facing an open arm and left to explore the maze for a single 5 min session with the experimenter out of view. After each trial, the floor was wiped clean with 10% alcohol.

The frequency of open entries (arm entry defined as all four paws into an arm) and the amount of time spent by the animals in open sections of the maze were recorded. We also calculated the % open entries (open entries/total entries x 100) and the % time spent in open arms (open time/300 x 100).We also recorded the rearing frequency and duration (all rearing occurred against the walls of the enclosed arms), the frequency of discrete behaviors, such as head dipping (exploratory movement of head/shoulders over sides of the maze), and the duration of grooming (species-typical sequence beginning with the snout, progressing to the ears, and ending with whole-body grooming). Each experiment was videotaped using a high-resolution video camera. These data were collected using X-Plo-Rat 2005 software (Taverna-Chaim and Morato, 2008).

2.8.Western blotting

After behavior testing, the animals were sacrificed by decapitation, and the hippocampus and amygdala were dissected and immediately frozen in liquid nitrogen and stored at 80˚C until analysis. The tissues were dissected and homogenized in lysis buffer according to a described previously protocol (Cardona- Gomez et al., 2004). Membranes were incubated overnight with the following primary antibodies: PHF-1 monoclonal antibody, which recognizes Tau pSer-396/404 and was donated by P. Davies (Feinstein Institute for Medical Research, Manhasset, NY); antiphospho-PHF-tau (pSer202/Thr205 Antibody (AT8) #MN1020, Thermo Scientific, 1:500); anti-NOS2 (C-11) Ms mAb (# Sc 7271, Santa Cruz Biotechnology, 1:100); rabbit polyclonal anti-COX2 (#AB15191, Abcam, 1:1000); rabbit phospho-p38 MAPK Thr180/ Tyr182 (# 9215, Cell Signaling, 1:1000); and tubulin (Mouse monoclonal anti-βIII tubulin antibody, #G712A, Promega, 1:10000) as a loading control. IRDye 800CW goat anti-mouse or rabbit (LICOR; diluted 1:10000) was used as a secondary probe. The blots were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences, United States).

2.9. β-Amyloid and IL-1β ELISA

Brain levels of soluble Aβ 1-40 and 1-42 were measured using ELISA. Soluble Aβ levels were measured by sandwich capture ELISA using Colorimetric BetaMark -βAmyloid x-40 (SIG-38954- Covance Laboratories) and x-42 ELISA (SIG-38956- Covance Laboratories) kits. IL-1β was measured with a Quantikine ELISA Mouse IL-1β (Cat. #MLB00C, R&D Systems, Minneapolis, USA) kit following the manufacturer's protocol at a protein concentration of 50 µg/mL.

2.10.Statistics

At least 3 mice were used for each histological study; 4-6 mice were used for each biochemical study; and 4-5 mice and 10-12 mice were used for EPM and MWM, respectively. Parametric data were evaluated with analysis of variance (ANOVA) to compare the 4 groups and then with Tukey's test for post hoc multiple comparison between-group analyses. Nonparametric data were evaluated using the Kruskal-Wallis test. The escape latency during the training and the transfer test was tested using two-way ANOVA followed by a Dunnett's post hoc test for multiple comparisons. The statistical analysis was performed using GraphPad Prism software (version 6.0), and the results were considered significant when $p \leq 0.05$. The values are expressed as the means ± SEM.

3. Results

3.1. Linalool treatment reversed spatial memory impairment in old 3xTg-AD mice

The MWM test is one of the most widely accepted behavioral tests for monitoring spatial learning and memory skills, which primarily depend on the hippocampus. Non-Tg mice

treated with vehicle and 3xTg-AD mice treated with vehicle or linalool were assessed. At the beginning of the test, all groups showed a similar performance during trials 1 and 2 on the first day. The average latency to the target platform for all groups during the 5 days of training is shown in Fig. 2 a. As previously demonstrated, 3xTg-AD mice exhibit a higher latency to locate the platform in the MWM test because of the synaptic dysfunction and long-term potentiation (LTP) deficits in these animals (Cantanelli et al., 2014). Interestingly, spatial learning abilities were restored in 3xTg-AD mice treated with linalool for 3 months. Starting at the third trial, treated 3xTg-AD mice were similar to Non-Tg mice treated with vehicle (Fig. 2 a). During the retention test, the hidden platform was removed for measurements of the hippocampus-dependent memory 48 h after the final learning trial. Non-Tg mice and the 3xTg-AD mice treated with linalool exhibited a higher retention performance than that of the 3xTg-AD vehicle-treated mice (Fig. 2 b). The linalool-treated 3xTg-AD mice crossed the platform a significantly higher number of times, spent more time reaching the platform, and swam a longer distance in the platform location than did the vehicle-treated 3xTg-AD mice; these values were similar to those of the Non-Tg mice (Fig. 2 c-e). Representative images of the travel routes during the retention test are provided in Fig. 2 f, which shows that 3xTg-AD animals treated with linalool remained closer to the hidden platform than did the 3xTg-AD animals treated with vehicle. Thus, the linalool treatment successfully reversed spatial memory impairment. The visible test did not reveal visual, motor or motivational deficits (Fig. 2 g).

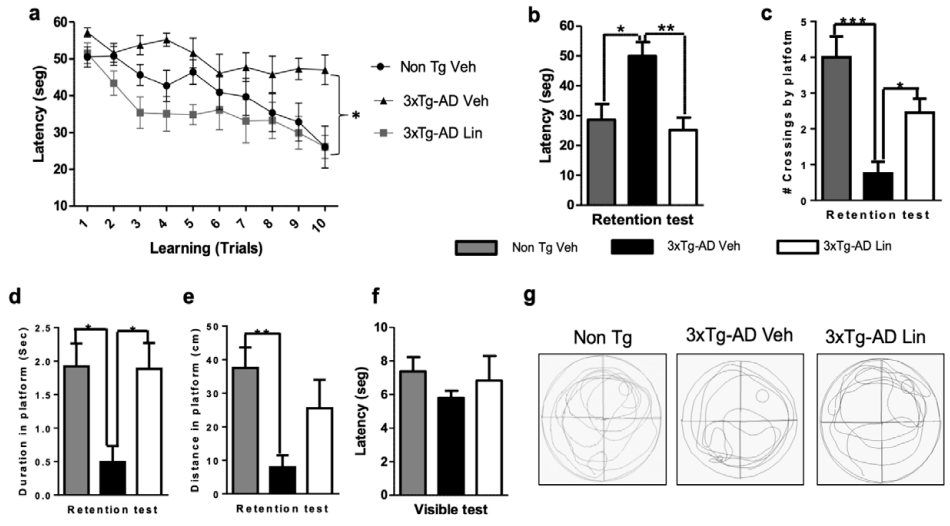


Fig. 2. Linalool treatment prevented spatial memory impairment in 3xTg- AD mice. a) Mean latency to reach the hidden platform during the spatial learning task. b) Latency, c) number of crossings, d) time spent reaching the platform and e) distance to reach the platform in the retention test. f) Representative images of the route of travel during the retention test. g) No differences were detected between the experimental groups in the visual, motor or motivational skills of the animals during the visible test. The data are expressed as the means ± SEM. n: 8-16. *Difference between the 3xTg-AD vehicle-treated group and the other groups *p<0.05.

3.2. Anxiolytic activity after oral administration of linalool in aged 3xTg-AD mice

We analyzed the effect of linalool on anxiety using the EPM test (Campos et al., 2013). Our data show a significant increase in the percentage of open-arm entries and the time spent in the open arms when comparing the 3xTg-AD mice treated with linalool to the Non-Tg and 3xTg-AD vehicle-treated mice, which rarely visited the open arms (Fig. 3 a-b). Additionally, 3xTg-AD vehicle mice showed a trend towards an increased number of grooming events and a significantly long amount of time spent grooming compared to those of the Non-Tg mice and the 3xTg-AD linalool-treated mice (Fig. 3 c-d). The number of occurrences of and time spent head-dipping were reduced in 3xTg-AD mice; in 3xTg-AD linalool treated mice, these values were restored to levels similar to those of the Non-Tg control mice (Fig. 3 e-f). This head-dipping behavior was inversely proportional to the number of rearing and the time spent rearing, which predominantly occurred in the closed arms. Therefore, rearing was significantly lower in the linalool-treated 3xTg-AD mice than in the other experimental groups (Fig. 3 g-h).

3.3. Aged 3xTg-AD linalool-treated mice show reduced β amyloidosis

3xTg-AD vehicle-treated mice showed the typical amyloid deposition found in the Non-Tg mice (Oddo et al., 2003), and this deposition was significantly reduced in the hippocampus, entorhinal cortex and amygdala of the 3xTg-AD linalool-treated mice (Fig. 4 a-e). The linalool treatment did not completely eliminate βamyloidosis in the 3xTg-AD mice. The decrease in β-amyloid levels was accompanied by a significant reduction in β-amyloid 1-40 and 1-42 protein levels in the hippocampal lysates of the linalool-treated 3xTg-ADmice compared to the vehicle-treated 3xTg-ADmice (Fig. 4 f-g). Together, these data show that cerebral amyloidosis, including amyloid deposits and b-amyloid peptide abundance, was delayed by the oral administration of linalool in old 3xTg-AD mice.

3.4. Linalool treatment ameliorated tau hyperphosphorylation in aged 3xTg-AD mice

Several abnormal tau hyperphosphorylation sites in AD have been identified using the increased tau aggregation at 18 months in the 3xTg-AD model (Oddo et al., 2003) We explored the effect of linalool on tau pathology in the brains of old 3xTg-AD mice using AT-8 immunoreactivity. 3xTg-AD mice treated with linalool displayed a significant decrease in pair helical filaments (PHFs) in the CA1 area, the subiculum and the amygdala but not in the EC. These findings were similar to the AT-8 immunoreactivity observed in the Non-Tg vehicle-treated mice (Fig. 5 e). We also found a significant reduction in PHF-1 protein levels in the hippocampal lysates from linalool-treated 3xTg-AD mice compared with vehicle-treated mice, suggesting a reduction in NFTs (Fig. 5 f).

3.5. Linalool decreased the inflammatory response in old 3xTg-AD mice

Chronic activation of glial cells around amyloid plaques is associated with AD pathophysiology via the production of numerous neurotoxic reactants, proinflammatory

cytokines, and immunostimulatory molecules. Reactive gliosis and increased expression of proinflammatory cytokines have been demonstrated in old transgenic mice with a model of cerebral amyloid deposition (Birch et al., 2014). To test whether linalool has an immunomodulatory effect on 3xTg-AD mice, we examined astrogliosis and microgliosis by evaluating GFAP and Iba1 immunoreactivity, respectively. Our data showed a significant increase in GFAP immunoreactivity in 3xTg-AD mice compared to the Non-Tg mice; however, linalool treatment significantly reduced the GFAP immunoreactivity in the CA1 hippocampal area, in the EC and in the amygdala compared to that in 3xTg-AD vehicle-treated mice (Fig. 6 a-e). We did not observe changes in the subiculum (Fig. 6 a, c).

Microglial activation is an AD hallmark associated with amyloidosis and tauopathy (Lee et al., 2013). We found that the linalool-treated 3xTg-AD mice displayed microglial immunoreactivity that was significantly decreased in the CA1 area, in the subiculum and in the amygdala compared to that in the vehicle-treated 3xTg-AD mice (Fig. 7 a-e). However, we did not find changes in the EC (Fig. 7 d). Microglial activation was related to βA immunolabeling (Fig. 8 a) and to the hippocampal upregulation of proinflammatory cytokines and the levels of associated marker proteins, such as IL-1b, iNOS, COX-2, and p38 MAPK, which were significantly upregulated in untreated 3xTg-AD mice compared to the Non-Tg group. This inflammatory response was reversed by linalool treatment (Fig. 8 b-e) (p

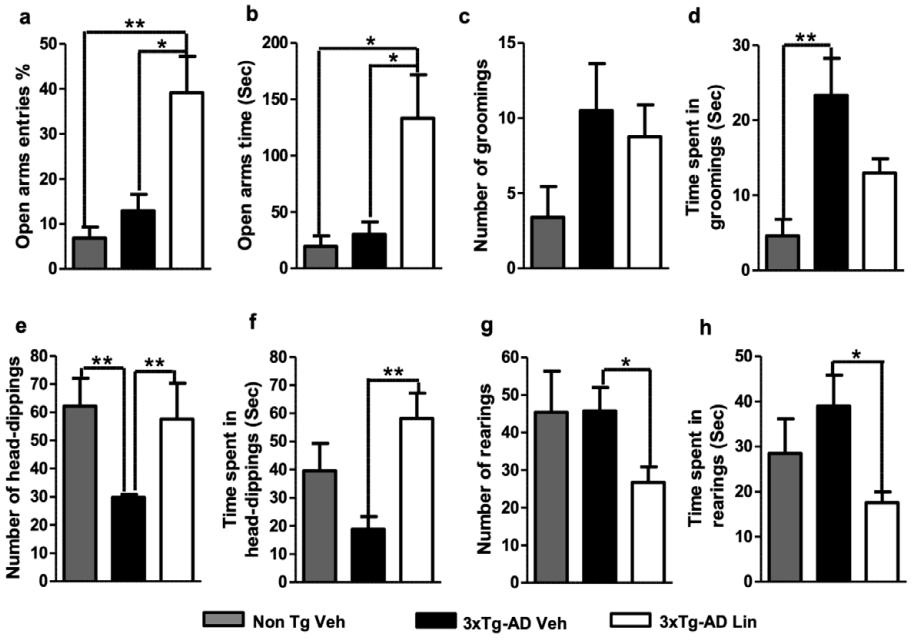


Fig. 3. Anxiolytic effect of linalool oral administration on 3xTg-AD mice. a-b) Relative frequency of open arm entries and the time spent in the open arms. c-d) Number of grooming behaviors and the time spent grooming. e-f) Number of head-dipping behaviors and the time spent head-dipping. g-h) Number of rearing actions and the time spent rearing at the end of the EPM test performed two weeks after the final dose of vehicle or linalool. The data are expressed as the means ± SEM. n: 4-5. *p<0.05.

< 0.05 - p < 0.001), confirming previous reports of the anti-inflammatory effects of linalool (Huo et al., 2013; Li et al., 2015).

4. Discussion

Oral administration of the monoterpene linalool ameliorated the histopathological hallmarks of AD and reversed the associated cognitive and emotional deficits in aged triple transgenic AD model mice. Our data suggests that linalool could be a pharmacological therapy for attenuating the neurotoxicity in neurodegenerative diseases. Few studies have reported linalool-mediated neuroprotection. Linalool protects against glucose/serum deprivation (GSD) in PC12 cells (Alinejad et al., 2013) and against acrylamide (ACR)-induced neurotoxicity in Wistar rats, increasing the glutathione (GSH) content while decreasing the ACR-induced lipid peroxidation in rat brain tissue (Mehri et al., 2014). However, other studies show that monoterpenes such as thymol and carvacrol and its derivates, inhibit acetylcholinesterase activity *in vitro* more strongly than linalool does (Jukic et al., 2007; Perry et al., 2000),

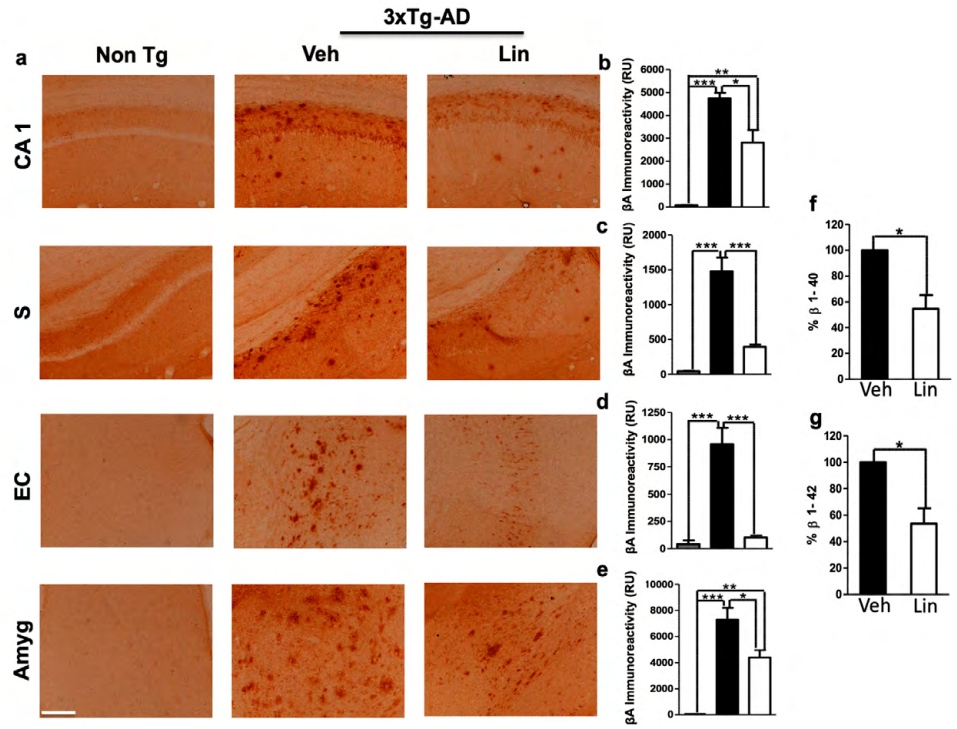


Fig. 4. 3xTg-AD linalool-treated mice show reduced b-amyloidosis. a) Representative images of bA (anti-bA 6E10) immunoreactivity in the CA1 area, the subiculum, the EC and the amygdala of vehicle- and linalool-treated 3xTg-AD and vehicle-treated Non-Tg mice at 21-24 months of age. Magnification: 10x; scale bar: 50 µm b) The values in the bar graph are expressed in densitometric relative units (RU) of bA immunoreactivity in the CA1 area, c) he subiculum, d) the EC and e) the amygdala. f) The relative βA 1-40 and g) βA 1-42 fragment levels were analyzed in hippocampal lysates by ELISA. Veh: vehicle (Saline solution); Lin: Linalool; CA1 and S: subiculum of the hippocampus; EC: entorhinal cortex; Amyg: amygdala. The data are expressed as the means ± SEM. n: 4-5. *p<0.05; **p<0.01; ***p<0.001.

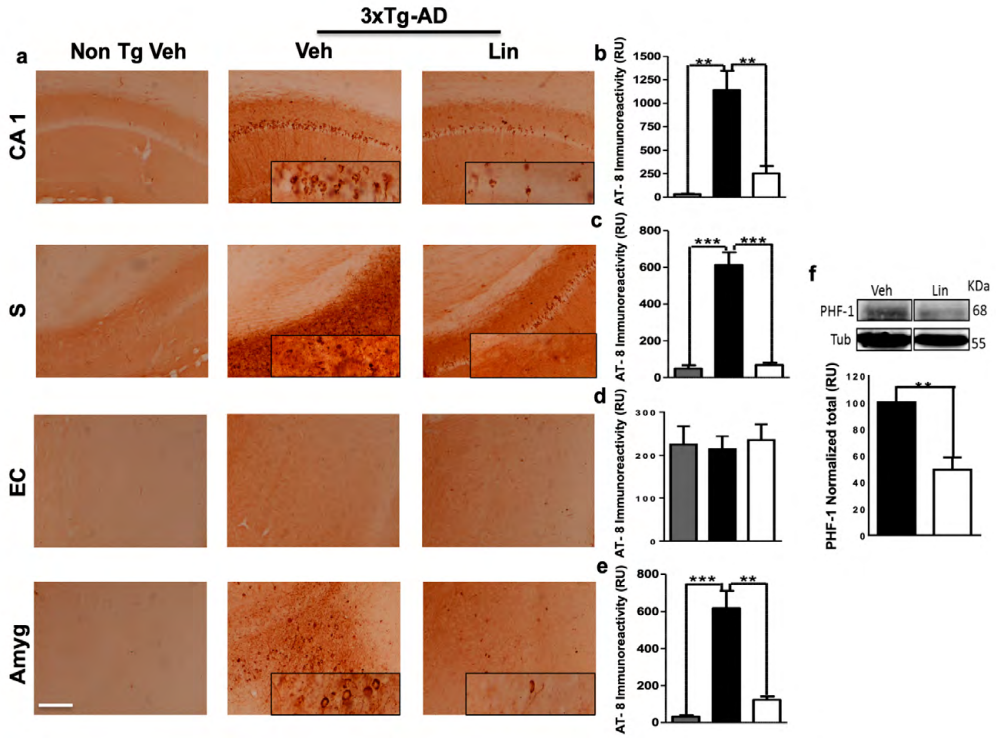


Fig. 5. Linalool treatment attenuated tau hyperphosphorylation in old 3xTg-AD mice. a) Representative images of AT8 (anti-tau pSer202/Thr205) immunoreactivity in the CA1 area, the subiculum, the EC and the amygdala of Non-Tg and 3xTg-AD mice treated with vehicle or linalool. Magnification: 10x; scale bar: 50 µm b) The values in the bar graph are expressed in densitometric relative units (RU) of AT8 immunoreactivity in the CA1 area, c) the subiculum, d) the EC and e) the amygdala. f) Representative bands and densitometric intensities of PHF-1 in hippocampal lysates. Tubulin was used as a loading control. Veh: vehicle (Saline solution); Lin: Linalool; CA1 and S: subiculum of the hippocampus; EC: entorhinal cortex; Amyg: amygdala. The data are expressed as the means ± SEM. n:4. *p<0.05; **p<0.01; ***p<0.001.

suggesting that linalool does not interfere with impaired cholinergic function. Also, thymol and carvacrol enhanced the performance on the MWM test after the injection of βA peptide in rat brains (Azizi et al., 2012). While linalool impaired the acquisition of long-term memory without interfering with short-term memory in rats in an object recognition task. This effect was associated with an antagonist capacity, similar to a glutamate antagonist (Coelho et al., 2011). Nevertheless, higher doses (i.p. 50-100 mg/kg) of linalool were used than those in our study (v.o. 25 mg/kg), which could explain our divergent results. Therefore, although linalool has been suggested to be a neuroprotective agent, its potential therapeutic role in AD has not been demonstrated previously.

EPM is one of the most widely used tests to measure anxiety and is based on the natural tendency of rodents to explore novel environments and their innate avoidance of unprotected, bright, and elevated places (Espejo et al., 1997). These behaviors can be regulated by the amygdala, a region of the limbic system involved in a variety of emotional and cognitive

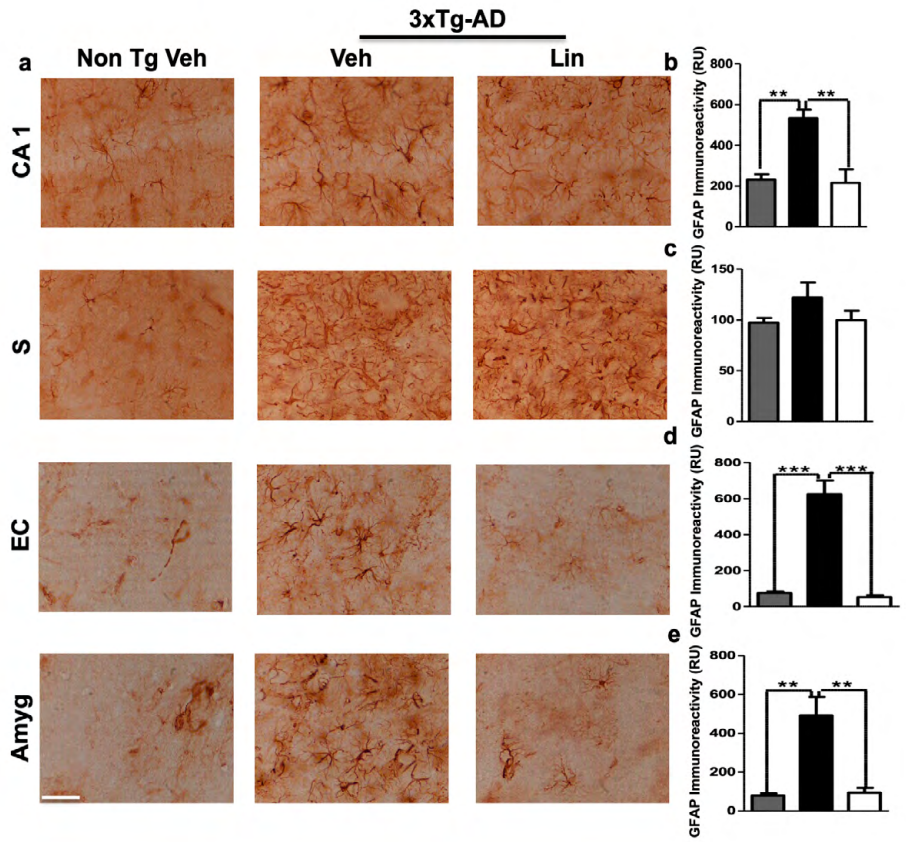


Fig. 6. Linalool decreased astrogliosis in old 3xTg-AD mice. a) Representative images of GFAP immunoreactivity in the CA1 area, the subiculum, the EC and the amygdala of Non- Tg and 3xTg-AD mice treated with vehicle or Linalool. Magnification: 40x. b) The values in the bar graphs are expressed in densitometric relative units (RU) of GFAP immunoreactivity in the CA1 area, c) the subiculum, d) the EC and e) the amygdala. Veh: vehicle (Saline solution); Lin: Linalool; CA1 and S: subiculum of the hippocampus; EC: entorhinal cortex; Amyg: amygdala. The data are expressed as the means ± SEM. n : 3-4. **p<0.01; ***p<0.001.

functions (Campos et al., 2013). Interestingly, our data showed that linalool-treated 3xTg-AD mice exhibited an increased frequency of entry into the open arms, increased head-dipping and reduced grooming and rearing frequencies compared with those of vehicle-treated 3xTg-AD mice. These results suggest reduced anxiety or a "risk assessment" behavior when the mice visit the open arms of the maze (Walf and Frye, 2007), which is unexpected considering that these results were obtained nine days after the final dose of linalool. Our data confirm the results of previous studies reporting that inhaled linalool has anxiolytic properties, increases social interaction, and decreases aggressive behavior (de Almeida et al., 2009; Linck et al., 2010). Although, other study did not report any difference in the number of entries to the open arms using 125 mg/kg linalool (Cline et al., 2008).

Linalool also reduced b-amyloidosis and tauopathy in the hippocampus (CA1, subiculum) and amygdala of aged 3xTg-AD linalool-treated mice compared to untreated mice. These data represent the first evidence that regular administration of linalool can prevent age-

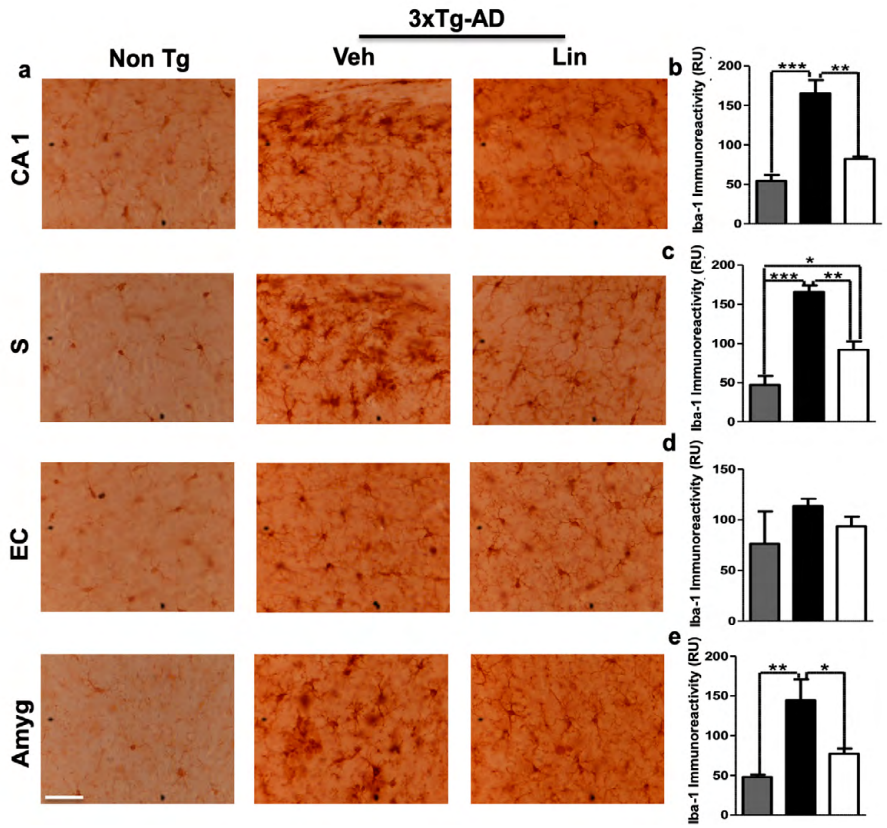


Fig. 7. Linalool ameliorated microgliosis in 3xTg-AD mice. a) Representative images of GFAP immunoreactivity in the CA1 area, the subiculum, the EC and the amygdala of Non-Tg and 3xTg-AD mice treated with vehicle or linalool. Magnification: 40x. b) The values in the bar graphs are expressed in densitometric relative units (RU) of Iba1 immunoreactivity in the CA1 area, c) the subiculum, d) the EC and e) the amygdala. Veh: vehicle (Saline solution); Lin: Linalool; CA1 and S: subiculum of the hippocampus; EC: entorhinal cortex; Amyg: amygdala. The data are expressed as the means ± SEM. n:3-4. *p<0.05 **p 0.01; ***p<0.001.

related cognitive impairments and β-amyloid accumulation in 3xTg-AD mice. The decreased b-amyloid level was accompanied by a reduction in β-amyloid 1-40 and 1-42 protein levels. These data are supported by studies, where a mixture of linalool and the monoterpene 2,3,4,4-tetramethyl-5- methylenecyclopent-2-enone led to a significant improvement in the reduction in brain-soluble Aβ 40 (Videira et al., 2014). Linalool is able to cross the blood-brain barrier (Cheng et al., 2015); however, the exact molecular mechanism of the linalool-mediated reduction in b-amyloid needs further study. Tauopathy is detected in pyramidal neurons in CA1 at 15 months of age (Oddo et al., 2003), and in our homozygous mouse colony, it is strongly detected at 18 months of age (Castro-Alvarez et al., 2014). Tauopathy is associated with degenerative symptoms, including significant deficits in hippocampus-dependent cognitive task performance. However, we did not detect immunoreactivity in the EC, which is consistent with some (Mastrangelo and Bowers, 2008; Sabogal-Guaqueta et al., 2015) but not all (Khan et al., 2014) previous studies.

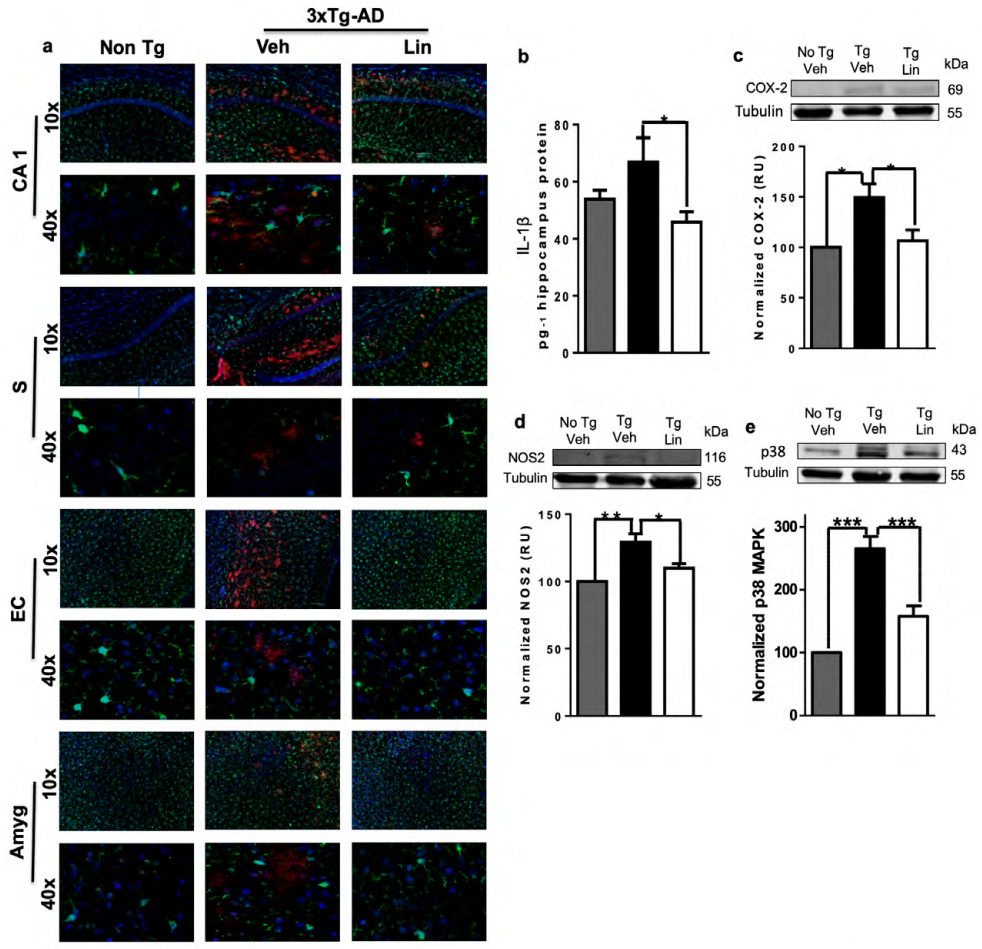


Fig. 8. Linalool reduced the proinflammatory response in 3xTg-AD mice. Morphological characterization showing nuclei stained with Hoechst (blue), b-amyloid stained with Alexa Fluor 594 phalloidin dye (red) and microglia visualized with Iba1 (green). Magnification, 10x and 60x. a) Representative images of CA1, subiculum, EC, and the amygdala. b) Quantification of IL-1b in hippocampal lysates. Representative bands and densitometric intensities of c) COX-2, d) NOS2 and e) p38 MAPK in hippocampal lysates. Veh: vehicle (Saline solution); Lin: Linalool; CA1 and S: subiculum of the hippocampus; EC: entorhinal cortex; Amyg: amygdala. The data are expressed as the means ± SEM. n: 4-5. *p<0.05; **p<0.01, ***p<0.001.

Hyperactived astrocytes and microglial proliferation accompanies βA deposition in the brains of AD model mice (Birch et al., 2014). In the present study, GFAP and Iba1 immunoreactivity was reversed in aged 3xTg-AD mice by oral treatment with linalool. In addition, linalool treatment reduced inflammatory markers, downregulating IL-1β and the downstream molecules p38 MAPK, COX-2 and iNOS. In previous studies, linalool reduced the serum and hepatic TNF-α, IL-6, iNOS and COX-2 expression by inhibiting NF-kB activation (Li et al., 2014). Moreover, linalool dramatically inhibited the LPS-induced phosphorylation of ERK, JNK, and p38 in RAW 264.7 cells (Huo et al., 2013). Linalool also has antioxidant properties and shows protective effects against hydrogen peroxide induced oxidative stress in brain

tissue (Celik and Ozkaya, 2002), which together could lead to fewer β-amyloid plaques and reduced tauopathy. Together, these findings suggest that monoterpenes, such as linalool, or the essential oils of several aromatic plants rich in this compound could be beneficial for AD patients or could be used as a chemical basis for the further development novel drugs to treat tauopathies.

5. Conclusion

In summary, our findings suggest that oral administration of linalool at an advanced stage of AD in 3xTg-AD model mice reversed the histopathological hallmarks of AD and restored cognitive and emotional functions. Thus, linalool may be a good candidate for further preclinical studies and future translational studies on AD.

Acknowledgments

The authors thank the Cellular and Molecular Neurobiology Area of the Neuroscience Group of Antioquia, the Group of Bioactive Substances, Professor Jose Ramirez from the Group of Immunomodulation of University of Antioquia and Professor Marisol Lamprea from the Neuroscience Laboratory at National University of Colombia for their scientific and technical support during the experiments. This research was funded by grants from COLCIENCIAS # 11565740581 (GPC-G), CODI University of Antioquia, Young Investigator Programme 2011e2012 Colciencias (AMS-G) and Project 1 R01 AG029802-01 NIA/NIH, Subcontract 2011e2012 (GPC-G). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

Alinejad, B., Ghorbani, A., Sadeghnia, H.R., 2013. Effects of combinations of curcumin, linalool, rutin, safranal, and thymoquinone on glucose/serum deprivation induced cell death. Avicenna J. Phytomed. 3, 321-328.

Anjos, P.J., Lima, A.O., Cunha, P.S., De Sousa, D.P., Onofre, A.S., Ribeiro, T.P., Medeiros, I.A., Antoniolli, A.R., Quintans-Junior, L.J., Santosa, M.R., 2013. Cardiovascular effects induced by linalool in normotensive and hypertensive rats.Z Naturforsch C 68, 181-190.

Association, A. s, 2014. Alzheimer's disease facts and figures. Alzheimer's Dementia 10.

Azizi, Z., Ebrahimi, S., Saadatfar, E., Kamalinejad, M., Majlessi, N., 2012. Cognitive enhancing activity of thymol and carvacrol in two rat models of dementia. Behav. Pharmacol. 23, 241-249.

Bassil, N., Grossberg, G.T., 2009. Novel regimens and delivery systems in the pharmacological treatment of Alzheimer's disease. CNS Drugs 23, 293-307.

Batista, P.A., Werner, M.F., Oliveira, E.C., Burgos, L., Pereira, P., Brum, L.F., Story, G.M., Santos, A.R., 2010. The antinociceptive effect of (-)-linalool in models of chronic inflammatory and neuropathic hypersensitivity in mice. J. Pain 11, 1222-1229.

Beier, R.C., Byrd 2nd, J.A., Kubena, L.F., Hume, M.E., McReynolds, J.L., Anderson, R.C., Nisbet, D.J., 2014. Evaluation of linalool, a natural antimicrobial and insecticidal essential oil from basil: effects on poultry. Poult. Sci. 93, 267-272.

Birch, A.M., Katsouri, L., Sastre, M., 2014. Modulation of inflammation in transgenic models of Alzheimer's disease. J. Neuroinflammation 11, 25.

Campos, A.C., Fogaca, M.V., Aguiar, D.C., Guimaraes, F.S., 2013. Animal models of anxiety disorders and stress. Rev. Bras. Psiquiatr. 35 (Suppl. 2), S101-S111.

Cantanelli, P., Sperduti, S., Ciavardelli, D., Stuppia, L., Gatta, V., Sensi, S.L., 2014. Age dependent modifications of AMPA receptor subunit expression levels and related cognitive effects in 3xTg-AD mice. Front. Aging Neurosci. 6, 200.

Cardona-Gomez, P., Perez, M., Avila, J., Garcia-Segura, L.M., Wandosell, F., 2004.Estradiol inhibits GSK3 and regulates interaction of estrogen receptors, GSK3, and beta-catenin in the hippocampus. Mol. Cell Neurosci. 25, 363-373.

Castro-Alvarez, J.F., Uribe-Arias, S.A., Kosik, K.S., Cardona-Gomez, G.P., 2014. Long and short-term CDK5 knockdown prevents spatial memory dysfunction and tau pathology of triple transgenic Alzheimer's mice. Front. Aging Neurosci. 6, 243.

Celik, S., Ozkaya, A., 2002. Effects of intraperitoneally administered lipoic acid, vitamin E, and linalool on the level of total lipid and fatty acids in guinea pig brain with oxidative stress induced by H2O2. J. Biochem. Mol. Biol. 35, 547-552.

Cline, M., Taylor, J.E., Flores, J., Bracken, S., McCall, S., Ceremuga, T.E., 2008. Investigation of the anxiolytic effects of linalool, a lavender extract, in the male Sprague-Dawley rat. AANA J. 76, 47-52.

Coelho, V.R., Gianesini, J., Von Borowski, R., Mazzardo-Martins, L., Martins, D.F.,Picada, J.N., Santos, A.R., Brum, L.F., Pereira, P., 2011. (-)-Linalool, a naturally occurring monoterpene compound, impairs memory acquisition in the object recognition task, inhibitory avoidance test and habituation to a novel environment in rats. Phytomedicine 18, 896-901.

Cheng, B.-H., Sheen, Lee-Yan, C, Shang-Tzen, 2015. Evaluation of anxiolytic potency of essential oil and S-(þ)-linalool from cinnamomum osmophloeum ct. linalool leaves in mice. J. Tradit. Complement. Med. 5, 27e34.

Cho, S.Y., Jun, H.J., Lee, J.H., Jia, Y., Kim, K.H., Lee, S.J., 2011. Linalool reduces the expression of 3-hydroxy-3-methylglutaryl CoA reductase via sterol regulatory element binding protein-2- and ubiquitin-dependent mechanisms. FEBS Lett. 585, 3289-3296.

Chung, M.J., Park, K.W., Kim, K.H., Kim, C.T., Baek, J.P., Bang, K.H., Choi, Y.M., Lee, S.J., 2008. Asian plantain (Plantago asiatica) essential oils suppress 3-hydroxy-3-methyl-glutaryl-co-enzyme A reductase expression in vitro and in vivo and show hypocholesterolaemic properties in mice. Br. J. Nutr. 99, 67-75.

de Almeida, E.R., Rafael, K.R., Couto, G.B., Ishigami, A.B., 2009. Anxiolytic and anticonvulsant effects on mice of flavonoids, linalool, and alpha-tocopherol presents in the extract of leaves of Cossus sicyoides L. (Vitaceae). J. Biomed. Biotechnol. 2009, 274740.

Dinda, B., Chowdhury, D.R., Mohanta, B.C., 2009. Naturally occurring iridoids, secoiridoids and their bioactivity. An updated review, part 3. Chem. Pharm. Bull.(Tokyo) 57, 765-796.

Elisabetsky, E., Brum, L.F., Souza, D.O., 1999. Anticonvulsant properties of linalool in glutamate-

related seizure models. Phytomedicine 6, 107-113.

Elisabetsky, E., Marschner, J., Souza, D.O., 1995. Effects of linalool on glutamatergic system in the rat cerebral cortex. Neurochem. Res. 20, 461-465.

Huo, M., Cui, X., Xue, J., Chi, G., Gao, R., Deng, X., Guan, S., Wei, J., Soromou, L.W., Feng, H., Wang, D., 2013. Anti-inflammatory effects of linalool in RAW 264.7macrophages and lipopolysaccharide-induced lung injury model. J. Surg. Res. 180, e47-54.

Ittner, L.M., Gotz, J., 2011. Amyloid-beta and tau-a toxic pas de deux in Alzheimer's disease. Nat. Rev. Neurosci. 12, 65-72.

Jukic, M., Politeo, O., Maksimovic, M., Milos, M., Milos, M., 2007. In vitro acetylcholinesterase inhibitory properties of thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. Phytother. Res. 21, 259-261.

Kalaria, R.N., Maestre, G.E., Arizaga, R., Friedland, R.P., Galasko, D., Hall, K., Luchsinger, J.A., Ogunniyi, A., Perry, E.K., Potocnik, F., Prince, M., Stewart, R., Wimo, A., Zhang, Z.X., Antuono, P., World Federation of Neurology Dementia Research, G, 2008. Alzheimer's disease and vascular dementia in developing countries: prevalence, management, and risk factors. Lancet Neurol. 7, 812-826.

Kang, J.H., Ryoo, N.Y., Shin, D.W., Trojanowski, J.Q., Shaw, L.M., 2014. Role of cerebrospinal fluid biomarkers in clinical trials for Alzheimer's disease modifying therapies. Korean J. Physiol. Pharmacol. 18, 447-456.

Khan, U.A., Liu, L., Provenzano, F.A., Berman, D.E., Profaci, C.P., Sloan, R., Mayeux, R., Duff, K.E., Small, S.A., 2014. Molecular drivers and cortical spread of lateral entorhinal cortex dysfunction in preclinical Alzheimer's disease. Nat. Neurosci. 17, 304-311.

Lee, D.C., Rizer, J., Hunt, J.B., Selenica, M.L., Gordon, M.N., Morgan, D., 2013. Review: experimental manipulations of microglia in mouse models of Alzheimer's pathology: activation reduces amyloid but hastens tau pathology. Neuropathol. Appl. Neurobiol. 39, 69-85.

Letizia, C.S., Cocchiara, J., Lalko, J., Api, A.M., 2003. Fragrance material review on linalool. Food Chem. Toxicol. 41, 943-964.

Li, J., Zhang, X., Huang, H., 2014. Protective effect of linalool against lipopolysaccharide/D-galactosamine-induced liver injury in mice. Int. Immunopharmacol. 23, 523-529.

Li, Y., Lv, O., Zhou, F., Li, Q., Wu, Z., Zheng, Y., 2015. Linalool inhibits LPS-induced inflammation in BV2 microglia cells by activating Nrf2. Neurochem. Res. 40, 1520-1525.

Linck, V.M., da Silva, A.L., Figueiro, M., Caramao, E.B., Moreno, P.R., Elisabetsky, E., 2010. Effects of inhaled linalool in anxiety, social interaction and aggressive behavior in mice. Phytomedicine 17, 679-683.

Linck, V.M., da Silva, A.L., Figueiro, M., Piato, A.L., Herrmann, A.P., Dupont Birck, F., Caramao, E.B., Nunes, D.S., Moreno, P.R., Elisabetsky, E., 2009. Inhaled linalool induced sedation in mice. Phytomedicine 16, 303-307.

Mastrangelo, M.A., Bowers, W.J., 2008. Detailed immunohistochemical characterization of temporal and spatial progression of Alzheimer's disease-related pathologies in male triple-transgenic mice. BMC Neurosci. 9, 81.

Mehri, S., Meshki, M.A., Hosseinzadeh, H., 2014. Linalool as a neuroprotective agent against acrylamide-induced neurotoxicity in Wistar rats. Drug Chem. Toxicol. 1-5.

Mitic-Culafic, D., Zegura, B., Nikolic, B., Vukovic-Gacic, B., Knezevic-Vukcevic, J., Filipic, M., 2009. Protective effect of linalool, myrcene and eucalyptol against tbutyl hydroperoxide induced genotoxicity in bacteria and cultured human cells. Food Chem. Toxicol. 47, 260-266.

Nascimento, S.S., Camargo, E.A., DeSantana, J.M., Araujo, A.A.S., Menezes, P.P., Lucca, W., Albuquerque, R.L.C., Bonjardim, L.R., Quintans, L.J., 2014. Linalool and linalool complexed in beta-cyclodextrin produce anti-hyperalgesic activity and increase Fos protein expression in animal model for fibromyalgia. Naunyn-Schmiedebergs Archives Pharmacol. 387, 935-942.

Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kayed, R., Metherate, R., Mattson, M.P., Akbari, Y., LaFerla, F.M., 2003. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron 39, 409-421.

Paxinos, G., Franklin, K.B.J., 2004. The Mouse Brain in Stereotaxic Coordinates.Elsevier Academic Press.

Perry, N.S., Houghton, P.J., Theobald, A., Jenner, P., Perry, E.K., 2000. In-vitro inhibition of human erythrocyte acetylcholinesterase by salvia lavandulaefolia essential oil and constituent terpenes. J. Pharm. Pharmacol. 52, 895-902.

Reitz, C., Mayeux, R., 2014. Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers. Biochem. Pharmacol. 88, 640-651.

Sabogal-Guaqueta, A.M., Munoz-Manco, J.I., Ramirez-Pineda, J.R., Lamprea- Rodriguez, M., Osorio, E., Cardona-Gomez, G.P., 2015. The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice. Neuropharmacology 93C, 134-145.

Silva Brum, L.F., Emanuelli, T., Souza, D.O., Elisabetsky, E., 2001. Effects of linalool on glutamate release and uptake in mouse cortical synaptosomes. Neurochem. Res.26, 191-194.

Tabassum, R., Vaibhav, K., Shrivastava, P., Khan, A., Ahmed, M.E., Ashafaq, M., Khan, M.B., Islam, F., Safhi, M.M., Islam, F., 2015. Perillyl alcohol improves functional and histological outcomes against ischemia-reperfusion injury by attenuation of oxidative stress and repression of COX-2, NOS-2 and NF-kappaB in middle cerebral artery occlusion rats. Eur. J. Pharmacol. 747, 190-199.

Taverna-Chaim, K., Morato, S., 2008. X-plo-rat 2005 1.1.0. Universidade de Sao Paulo, Ribeirao Preto. Videira, R., Castanheira, P., Graos, M., Resende, R., Salgueiro, L., Faro, C., Cavaleiro, C., 2014. Dose-dependent inhibition of BACE-1 by the monoterpenoid 2,3,4,4- tetramethyl-5-methylenecyclopent-2-enone in cellular and mouse models of Alzheimer's disease. J. Nat. Prod. 77, 1275-1279.

Walf, A.A., Frye, C.A., 2007. The use of the elevated plus maze as an assay of anxiety related behavior in rodents. Nat. Protoc. 2, 322-328.

Wu, Q., Yu, L., Qiu, J., Shen, B., Wang, D., Soromou, L.W., Feng, H., 2014. Linalool attenuates lung inflammation induced by Pasteurella multocida via activating Nrf-2 signaling pathway. Int. Immunopharmacol. 21, 456-463.

[a]Neuroscience Group of Antioquia, Cellular and Molecular Neurobiology Area - School of Medicine, SIU, University of Antioquia, Calle 70 # 52-21, Medellín, Colombia

[b]Institute of Biology, Faculty of Exact and Natural Sciences, University of Antioquia, Medellín, Colombia

[c]Grupo de Investigación en Sustancias Bioactivas, Facultad de Ciencias Farmacéuticas y Alimentarias, Universidad de Antioquía UdeA, Calle 70 No, 52-21,Medellin, Colombia

[d]Department of Systems Engineering, University of Antioquia, Medellín, Colombia

CHAPTER 3

Changes in the hippocampal and peripheral phospholipid profiles are associated with neurodegeneration hallmarks in a long-term global cerebral ischemia model: Attenuation by Linalool

Angélica María Sabogal-Guáqueta[a], Rafael Posada-Duque[a, b], Natalie Charlotte Cortes[c], Julian D. Arias-Londoño[d], Gloria Patricia Cardona-Gómez[a]

Neuropharmacology 135 (2018) 555-571. doi: 10.1016/j.neuropharm.2018.04.015

Abstract

Phospholipid alterations in the brain are associated with progressive neurodegeneration and cognitive impairment after acute and chronic injuries. Various types of treatments have been evaluated for their abilities to block the progression of the impairment, but effective treatments targeting long-term poststroke alterations are not available. In this study, we analyzed changes in the central and peripheral phospholipid profiles in ischemic rats and determined whether a protective monoterpene, Linalool, could modify them. We used an *in vitro* model of glutamate (125 µM) excitotoxicity and an *in vivo* global ischemia model in Wistar rats. Linalool (0.1 µM) protected neurons and astrocytes by reducing LDH release and restoring ATP levels. Linalool was administered orally at a dose of 25 mg/kg every 24 h for a month, behavioral tests were performed, and a lipidomic analysis was conducted using mass spectrometry. Animals treated with Linalool displayed faster neurological recovery than untreated ischemic animals, accompanied by better motor and cognitive performances. These results were confirmed by the significant reduction in astrogliosis, microgliosis and COX-2 marker, involving a decrease of 24:0 free fatty acid in the hippocampus. The altered profiles of phospholipids composed of mono and polyunsaturated fatty acids (PC 36:1; 42:1 (24:0/18:1)/LPC 22:6)/LPE 22:6) in the ischemic hippocampus and the upregulation of PI 36:2 and other LCFA (long chain fatty acids) in the serum of ischemic rats were prevented by the monoterpene. Based on these data, alterations in the central and peripheral phospholipid profiles after long-term was attenuated by oral Linalool, promoting a phospholipid homeostasis, related to the recovery of brain function.

Keywords: Ischemia, phospholipids, neurodegeneration hallmarks, linalool, functional recovery.

Changes in the hippocampal and peripheral phospholipid profiles are associated with neurodegeneration hallmarks in a long-term global cerebral ischemia model: Attenuation by Linalool

1. Introduction

Cerebral ischemia is the third leading cause of death and the first cause of acquired disability worldwide (Bousser, 2012). Ischemia causes significant sensorimotor and cognitive impairments, as well as physical and mental disabilities and other complications, such as post-stroke dementia, depression, falls, fractures, and epilepsy, altering the quality of life of patients and their families (Rothwell et al., 2011). Ischemic stroke is caused by the occlusion of cerebral blood vessels, which deprive brain cells of the oxygen and glucose required for their function. The resulting energy deficiency perturbs mitochondrial ATP synthesis and increases the generation of free radicals, leading to oxidative stress and lipid peroxidation. Concomitantly, the activation of excitatory neurotransmitters such as dopamine and glutamate induce intracellular calcium overload, metabolic dysfunction and acidosis (Moskowitz et al., 2010). An increase in the intracellular calcium concentration activates sphingomyelinases and phospholipases A2, C and D which, in turn, promote the release of second messengers such as diacylglycerol (DAG), phosphatidic acid and arachidonic acid (ArAc), which are involved in inflammation, excitotoxicity and other cell death pathways (Phillis and O'Regan, 2004; Tian et al., 2009).

In general, lipids are complex, diverse and their functions rely on their cellular localization. Lipids are involved in maintaining the membrane structure and may act as metabolic signaling molecules and neuromodulators in the central nervous system (CNS) (Martinez-Gardeazabal et al., 2017). Specifically, phospholipids are critical components of the endolysosomal system and modulate the trafficking of neural cells and the release of neurotransmitters; in patients with a brain disease, modifications to the fatty acid composition of phospholipids affect their homeostasis and function. For example, many phenomena observed during brain ischemia and reperfusion are accounted for by damage to membrane lipids, specifically lipolysis during ischemia and radical mediated peroxidation of polyunsaturated fatty acids (PUFAs) during reperfusion (Rabiei et al., 2012). However, the role of phospholipids in the long-term post-stroke neuropathogenesis and mechanisms to prevent this disorder are not clearly understood.

The development of new strategies for the prevention and treatment of stroke has grown considerably over the last few years. The main focus of the therapy relies on decreasing the acute disability of stroke through the use of thrombolytic drugs (e.g., recombinant tissue plasminogen activator) to limit the infarct size and improve the outcome, but has the difficulty of only being able to be administered in a small therapeutic window (Schulz, 2013). Currently, several natural products have been used for neuroprotective purposes. Among them, Linalool exhibits different biological properties that could be used to treat cerebrovascular diseases.

Linalool (Lin, 3,7-dimethyl-1,6-octadien-3-ol) is a monoterpene alcohol that is naturally found in some plants (e.g., anise, pepper and fennel), is useful for attracting pollinators in the natural environment (Raguso, 2016), and has been used in the cosmetic and medical industries as a flavoring. Linalool has numerous biological activities, including anti-neoplastic (Miyashita and Sadzuka, 2013), anti-inflammatory (Li et al., 2016), anti-leishmanicidal, antioxidant and antimicrobial activities (Park et al., 2012; Shi et al., 2016). Linalool was recently shown to exert effects on the CNS, including sedative, anti-nociceptive, anticonvulsant, anti-depressive and anxiolytic effects (Coelho et al., 2013; Guzmán-Gutiérrez et al., 2015). It has also been reported to modulate neurotransmission *in vitro* and *in vivo* models through its effect on the NMDA receptor (Elisabetsky et al., 1995). As shown in our recent study, Linalool also exerts neuroprotective effects on a triple transgenic AD mouse model by reversing the histopathological hallmarks and cognitive and emotional dysfunction (Sabogal-Guáqueta et al., 2016). Moreover, in a 2014 study by Mehri et al. of a rat model of acrylamide induced neurotoxicity, this molecule reduced lipid peroxidation in the brain tissue (Mehri et al., 2015).

In addition, the two-vessel occlusion (2-VO) animal model gives rise to ischemic cell change in a number of brain areas including the hippocampal CA1 subfield, striatum and neocortex (McBean and Kelly, 1998). Although, the infarct volume is not quantifiable in this model, because of the diffuse and variability pattern of the infarcted areas. 2-VO model reproduces neurological, motor and cognitive impairments at short and long-term post-ischemia in agreement with our previous studies (Becerra-calixto and Cardona Gómez, 2016; Gutiérrez Vargas et al., 2010; Villamil Ortiz and Cardona Gomez, 2015).

Therefore, considering the limited state of the art of monitoring phospholipids after stroke, in the present study, we used *in vitro* glutamate excitotoxicity model and *in vivo* 2-VO cerebral ischemia model to context the neural cell imbalance and to understand the affection on the hippocampal and peripheral phospholipid profile, also analyzing whether a Linalool treatment might modify these parameters.

2. Materials and methods

2.1. Animal procedures

All animal procedures were performed in accordance with the ARRIVE guidelines, the Guide for the Care and Use of Laboratory Animals, 8th edition, published by the National Institutes of Health (NIH) and Colombian standards (law 84/1989 and resolution 8430/ 1993). These procedures were approved by the Ethics Committee for Animal Experimentation of the University of Antioquia, Medellin, Colombia.

2.2. Dissociated cortical neuron and astrocyte cultures

Cerebral cortices and hippocampi from Wistar rat embryos (E18) were dissected, trypsinized and cultured on multi-well plates precoated with poly-L-lysine (Sigma-Aldrich) in Neurobasal medium (GIBCO) containing B-27 supplement (Sigma Aldrich) and a penicillin-streptomycin antibiotic mixture (GIBCO) at 37°C in a 5% CO_2 humidified atmosphere for a maximum of 7 days (DIV 7) or 19 days (DIV 19) *in vitro* for immature and mature cultures, respectively. Neurons that had been isolated and dissociated as described above were plated at a density of 500 cell/mm2 (equivalent to 100,000 cells per well) in 24-well plates for immunofluorescence and cytotoxicity assays or at a density of 250 cell/mm2 (50,000 cells per well) in 96-well plates. On the other hand, the cortices and hippocampi of neonate Wistar rats (PN1e2) were dissected, trypsinized, dissociated, and cultured in 75-cm2 flasks at 37 C and 5% CO_2 to obtain astrocytes (Posada-Duque et al., 2015). The cell confluence was observed at DIV10 and was approximately $4x10^6$ cells. Subsequently, astrocytes were subcultured at DIV 13-14, by treatment with 0.25% trypsin/EDTA (GIBCO) and replated in 12- or 24 well plates at densities of 7.5 $x10^4$ and 3.5 x 10^4 cells per well, respectively.

2.2.1.LDH release

Linalool was dissolved in DMSO (Dimethyl sulfoxide) and added to cultures of neurons or astrocytes at different concentrations (0.1 µM, 1 µM, 10 µM, 100 µM and/or 200 µM). Cytotoxicity was assessed by measuring the amount of lactate dehydrogenase (LDH) released from the cultures using a cytotoxicity detection kit (Cat. No. 11644793001-Roche Molecular Biochemicals, Indianapolis, IN, USA) as previously described (Posada-Duque et al., 2013). All experiments were performed three times in duplicate.

2.2.2.In vitro protection assays

Neuronal and astrocytes cultures from cortices and hippocampi were treated with 125 µM glutamate as toxic stimulus and glutamate buffer (pH: 7) as vehicle for 20 min and post-treated with Linalool (n:3-4) at concentration of 0.1 µM. We evaluated LDH release from mature neurons at DIV 19 and astrocytes at DIV 24, also the percentage of condensed nuclei under the same conditions using the following formula: [condensed nuclei/(condensed nuclei + normal nuclei)] x 100 (Posada-Duque et al., 2013). Analyses were performed for 30 neurons per treatment in each duplicate assay from at least three or four independent experiments.

2.2.3. ATP quantification assay

Homogenized cells were used to quantify ATP levels with the CellTiter-Glo Luminescent Cell Viability Assay (Promega Ref. G7571), according to manufacturer's protocol.

2.2.4. Determination of MDA levels

DIV 6 neurons were first pre-treated with Linalool at concentrations of 0.1 µM, 1 µM, 10 µM, or 100 µM, and DIV 7 neurons were treated with 125 µM glutamate as toxic stimulus and glutamate buffer (pH : 7) as vehicle for 20 min and post-treated with Linalool (n : 3-4) at concentration of 0.1 µM, 1 µM, 10 µM, or 100 µM. Also, mature neuronal and astrocytes cultures from cortices and hippocampi were post-treated with Linalool (n: 3-4). For the determination of the MDA (malondialdehyde) concentration (thiobarbituric acid reactive substances, TBARS), trichloroacetic acid and TBARS reagent were added to supernatants from neuron and astrocyte cultures, mixed and incubated in boiling water for 90 min. After cooling on ice, samples were centrifuged at 3000 x g for 10 min and the absorbance was read at 532 nm. Results were reported according to a tetraethoxypropane standard curve.

2.3.Animals and drug administration

Male Wistar albino rats from our in-house, pathogen-free colony in the vivarium at SIU (Sede de Investigacion Universitaria) (University of Antioquia, Medellin, Colombia) were maintained on a 12:12-h dark/light cycle and received food and water ad libitum. Special care was taken to minimize animal suffering and to reduce the number of animals used. Three-month-old rats weighing 400-450 g were used. Twenty-four rats per experimental group were used for neurological scoring in the motor and cognitive tests. Four to five rats per experimental group were used for the histological and biochemical assessments.

Rats were randomly divided into 4 groups as follows: 2 sham groups, animals underwent identical procedures except the occlusion of arteries. Sham control (sham ctr) administration of vehicle: PEG, Polyethylene glycol 4000,1% and sham Linalool (sham Lin) of Linalool 25 mg/kg/d. Two groups were subjected to 2VO ischemia denoted as the ischemic control (isc ctr; vehicle: PEG 1%) and ischemic Linalool (isc Lin; 25 mg/kg/d). All rats were administered an oral dose of Linalool or vehicle every 24 h for one month. Behavioral tests were performed during this period (Fig. 3a). Previously, 25 mg/kg Linalool had not shown toxicity (Api et al., 2016) and the interval between drug treatment and assays was selected based on previous *in vivo* studies (Mehri et al., 2015; Sabogal-Guáqueta et al., 2016).

2.4. Global cerebral ischemia (2VO)

Global cerebral ischemia was induced in male Wistar rats using a previously described modified two-vessel occlusion (2VO) method (Villamil Ortiz and Cardona Gomez, 2015). Briefly, the right common carotid artery (CCA) was permanently occluded using a 6.0-gauge nylon suture (Corpaul, Bogota, Colombia) and the left CCA was obstructed for 20 min using a vascular clip. After 20 min, the vascular clip was removed to allow reperfusion. Sham rats underwent the same procedure without the CCA occlusion. At 6 h after the completion of the surgery, a neurological test was performed to evaluate the rats' sensory and motor abilities.

2.4.1. Neurological evaluation

Neurological performance was evaluated from 6 h to 7 days after 2VO ischemia in each experimental group (n: 24). Neurological function was scored on an 18-point scale based on the Garcia test (Garcia JH, Wagner S, Liu KF, 1995). The neuroscore combined six different neurological tests: (1) spontaneous activity, (2) symmetry in limb movement, (3) forepaw outstretching, (4) climbing, (5) body proprioception, and (6) response to vibrissae touch. Each test received a maximum score of three points based on a set of predetermined criteria described by Garcia et al., in 1995 (Garcia JH, Wagner S, Liu KF, 1995). Scores for each test were summed with a highest possible score of 18 points indicating no neurological deficits and a lowest score of 3 points for animals with the most severe impairments. Neurological scoring was performed each day and in the same order and time for all rats.

2.4.2. Motor skill test

At 6 h post-ischemia and immediately after neurological testing, each rat (n: 18-21) was placed on a Rotarod device (IITC Inc. Life Science, Woodland Hills, CA, USA). Latency (seconds), distance (meters) and velocity (rpm: revolutions per minute) were recorded for each animal as it kept walking on the moving cylinder at a constant speed. The rod is a drum that is 7 cm in diameter, elevated 25 cm above the bottom of the apparatus, and equipped with a motor to control the rotation speed. Briefly, rats were trained for 2 days before 2VO global ischemia. The score was measured using the previously described parameters from 6 h to 7 days (Becerra- Calixto and Cardona-Gómez, 2016), and the animals that did not learn the rotarod task were excluded from this analysis.

2.4.3. Inclined plane test

The animal's ability to maintain postural stability was assessed using the inclined plane test (n: 24). The relative angle at which the rat could no longer maintain its position for 10 s was recorded as the final angle and it was considered a measure of functional impairment (Koc et al., 2015).

2.4.4. Morris water maze test

Nineteen days after ischemia, the animals were evaluated in the Morris water maze (MWM) (n: 18-19). The test was performed using a previously described method (Becerra-Calixto and Cardona-Gómez, 2016). Briefly, a black plastic tank was filled with water (22 ± 2°C), and visual cues around the room remained in a fixed position throughout the experiment. The hidden platform (12 cm diameter) was submerged 3 cm below the water level during spatial learning and 1.5 cm above the surface of the water during the visible session. Six sessions or trials were performed. Each session consisted of four successive subtrials (30 s inter-trial interval), and each subtrial began with the rat being placed pseudo-randomly in one of four starting locations. Then, the animals were provided with a 48 h retention period, followed by a probe trial of spatial reference memory, in which the animals were placed in the tank

without the platform for 90 s. The latency to reach the exact former location of the platform was recorded during the probe trial. Later, the platform was moved to a new location and the animals' ability to learn the new location was measured by determining the latency in 4 sessions conducted in the same manner as the learning phase. The latency to reach the platform was evaluated using a visible platform to control for any differences in visual-motor abilities or motivation between the experimental groups; the animals that could not perform this task were excluded. An automated system (Viewpoint, Lyon, France) recorded the behavior of the animals.

2.5. Histology

Twenty-four hours after the final behavioral test, animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (50 mg/kg) plus xylazine (20 mg/kg) and were perfused with a 0.9% saline solution and 4% paraformaldehyde (0.1MPBS, pH 7.4). Brains were removed and post-fixed with 4% paraformaldehyde at 4℃ for 48h, then incubated with sucrose and stored in a glycol-glycerol-based cryoprotectant at 20℃. The brains were sectioned (50 µm) with a Leica VT1000S vibrating blade microtome (Leica Microsystems, Germany). The ipsilateral hippocampus (main hippocampal side affected by the permanent occlusion of right common carotid artery) was analyzed at bregma -2.30mm (George Paxinos, 2006).

2.5.1. Immunohistochemistry

Coronal sections (50 µm) were permeabilized, blocked with 0.3% Triton X-100 and 1% BSA in PBS, and then probed with the following primary antibodies using a previously described protocol (Sabogal-Guáqueta et al., 2016): anti-NeuN (mouse monoclonal MAB377, Millipore, 1:500) anti-GFAP (monoclonal anti-glial fibrillary acidic protein, #G 3893, Sigma, 1:500), and anti-Iba1 (rabbit anti-Iba1 (ionized calcium binding adaptor molecule, #019-19741, Wako, 1:500). The tissues incubated in the absence of primary antibody did not display immunoreactivity. Quantification of immunoreactivity in the areas examined was determined using a 40x objective and was analyzed using Fiji ImageJ 1.45 software (NIH, USA). The images were modified to a binary system, and integrated densities (relative units) were obtained for each image. The background was automatically subtracted in each image for quantification of the relative intensity of immunostaining as previously we described in Gutiérrez-Vargas et al. (2015).

2.5.2. Immunofluorescence

Cultures were fixed with 4% formaldehyde prepared in a cytoskeleton buffer for 20 min. Autofluorescence was blocked with 50mM NH4Cl. Cells were permeabilized and blocked with PBS + 0.1% Triton X-100 and 1% fetal bovine serum for 1 h. Cultures were incubated with the following primary antibodies overnight at 4 C: MAP2 (1:2000, Sigma) or GFAP (monoclonal anti-glial fibrillary acidic protein, #G 3893, Sigma, 1:500). Alexa Fluor 488-conjugated anti-

mouse or anti-rabbit secondary antibodies (1:2000, Molecular Probes) were used as probes. Nuclei were stained with Hoechst 33258 (1:5000, Invitrogen), and the F-actin cytoskeleton visualized with Alexa Fluor 594-conjugated phalloidin dye.

Sections at the level of the bregma -2.32mm received the same treatment that cells. They were incubated overnight at 4 C with GFAP (anti-glial fibrillary acidic protein, G 3893, Sigma, Ms 1:500) and COX-2 antibodies (ab15191, rabbit anti-COX-2, Abcam, 1:500) diluted in PBS with Triton X-100 and BSA 0.3%. Then, sections were washed four times with 0.1MPBS and incubated for 90 min at room temperature with mouse Alexa Fluor 488- or Alexa Fluor 594- conjugated anti-rabbit secondary antibodies (1:1000; Molecular Probes, Eugene, OR). Sections were stained with Hoechst (1:1000, Sigma) for 15 min at room temperature. Neurons and tissue sections were washed four times with buffer, cover slipped using Gel Mount (Biomeda) and observed under an Olympus IX 81 epifluorescence microscope (Olympus, Japan) or DSU Spinning Disc confocal microscope (Olympus, Japan). Omission of the primary antibodies resulted in a lack of staining. The sections were photographed at 40x magnifications. Those images were analyzed evaluating the fluorescence intensity of GFAP and COX-2 immunostaining using Fiji ImageJ 1.45 (Gutiérrez-Vargas et al., 2015).

2.6. Lipid analyses

Total lipids were extracted from the hippocampus and serum using the Folch technique (Jordi Folch, 1957) with a mixture of 2mL of chloroform ($CHCl_3$) and 1mL of methanol (MeOH) in a 2:1 (v/v) ratio. Then, 0.005% butylated hydroxytoluene (BHT)was added, and this mixture was used to homogenize the hippocampus. Subsequently, 1mL of 0.9% NaCl was added, and the mixture was centrifuged at 3000 rpm for 3 min. The organic layer (lower layer) was removed and transferred to a new glass tube. Solvents were evaporated, and the extract was lyophilized to remove the excess humidity. Finally, the lipid composition was analyzed by mass spectrometry.

2.6.1. Mass spectrometry

An automated ESI-MS/MS approach was used and data acquisition and analysis were performed at the Kansas Lipidomics Research Center using an API 4000 TM and Q-TRAP (4000Qtrap) detection system, as described previously (Villamil-Ortiz et al., 2016). This protocol allowed the detection and quantification of low concentrations of the polar lipid compounds. Molecules were determined based on their mass/charge ratios, which were compared with the respective internal standard to determine which species of lipids were present in the evaluated extract: 0.30 nmol of 14:0 lysoPG, 0.30 nmol of 18:0 lysoPG, 0.30 nmol of di 14:0 PG, 0.30 nmol of 14:0 lysoPE, 0.30 nmol of 18:0 lysoPE, 0.60 nmol of 13:0 lysoPC, 0.60 nmol of 19:0 lysoPC, 0.60 nmol of di 12:0-PC, 0.60 nmol of di 24:1-PC, 0.30 nmol of 14:0 lysoPA, 0,30 nmol of 18:0 lysoPA, 0,30 nmol of di14:0 PA, 0.30 nmol of di 20:0 (phytanoyl) PA, 0.20 nmol of di 14:0 PS, 0,20 nmol of di Phy PS, 0,28 nmol of 16:0e18:0 PI,

and 0,10 nmol of di 18:0PI. The system detected a total of 12 different lipid species and their respective sub-species, which were identified by the number of carbons and degree of unsaturation of the chain. Lipid concentrations were normalized to the molar concentration across all species in each sample, and the final data are presented as the mean %Mol.

2.6.2. Profile of circulating FFAs

Lipids were extracted from the hippocampus using the Folch method. The solid-phase extraction (SPE) method described by Velasquez (Bermúdez-cardona and Velásquez-rodríguez, 2016) was used to separate cholesterol esters (CE), triglycerides (TG) and phospholipids (PL).

2.7. Statistical analysis

The sample sizes (n) used for statistical analyses correspond to the number of animals per experimental group. The behavioral test was performed on 18-24 animals/group. The escape latency during the hidden platform training sessions, transference, neurological score, rotarod and incline plane tests were analyzed using repeated-measures ANOVA. The latency in the retention test and histological and biochemical analysis were analyzed using ANOVA to compare the 4 groups and then with Tukey's test for post hoc multiple comparisons of the parametric data in between-group analyses. Nonparametric data were evaluated using the Kruskal- Wallis test. Analyses were performed using GraphPad Prism 6 software. Values are expressed as means ± SEM. Results were considered to be significant at $p < 0.05$ and $p < 0.01$. All sample groups were processed in parallel to reduce inter-assay variation.

The lipid levels in each sample were calculated by summing the total number of moles of all lipid species measured and then normalizing that total to %Mol. Comparisons between groups were assessed using either one-way ANOVA followed by the Tukey post hoc test or the Kruskal-Wallis test, depending on the homoscedasticity and normality of the experimental data. Multivariate statistics were performed using a principal component analysis (PCA) and a partial least squares discriminant analysis (PLS-DA) (Barker and Rayens, 2003). PLSDA was included because it is particularly suitable for the analysis of datasets with a small number of samples and a large number of variables. The PLS-DA was conducted using the routines described previously by our laboratory (Villamil-Ortiz et al., 2016). The data from the univariate and bivariate statistics are expressed as means ± the standard error of the mean. The statistical significance is indicated in the figures and tables.

3. Results

3.1. LDH release and ATP deficiency in response to glutamate excitotoxicity in neuron and astrocyte cultures were blocked by Linalool

We evaluated the effects of Linalool on mature cultures of neurons and astrocytes from

Changes in the hippocampal and peripheral phospholipid profiles are associated with neurodegeneration hallmarks in a long-term global cerebral ischemia model: Attenuation by Linalool

cortices and hippocampi subjected to glutamate toxicity. It has been implicated widely in the pathogenesis of a range of neurological diseases, including cerebrovascular diseases. To determine if Linalool is protective on primary cultures, we followed the experimental design presented in Fig. 1a and 2a. As shown in Fig. 1 b, f and 2 b, f in both type of neural cells, the treatment with 0.1 µM Linalool blocked glutamate induced cell death, and prevented the retraction of processes (MAP2+ or GFAP+, respectively) and actin cytoskeleton depolymerization induced by glutamate (Fig. 1 b, 1 f and 2 b, 2 f).

The measurement of LDH released into the extracellular medium was used to determine cell survival. We observed Linalool decreased LDH release (Fig. 1 c, g and 2 c, g) and also reduced the percentage of cells with condensed nuclei (Fig.1 d, h and 2 d, h) and recovered ATP levels (Fig. 1 e, i and 2 e, i). On the other hand, we observed reduction of lipid peroxidation with different concentrations of linalool and mainly in neurons and astrocytes from cortices but not from hippocampi (Suppl. Fig.1 a-d). These findings suggest that treatment with Linalool induces neuronal and astrocytic protection, maybe in a tissue differential-mode.

3.2. Post-ischemic neurological, motor and cognitive impairments were prevented by the oral Linalool treatment

Next, we validated the effect of Linalool on behavioral tests in a Wistar rat model of global cerebral ischemia model (Fig. 3 a). Ischemic rats exhibited significantly lower neurological scores than sham-operated rats (**$p < 0.01$; n: 24) (Fig. 3 b). However, ischemic rats treated with Linalool displayed significantly higher scores than the ischemic group and similar scores to the sham group (**$p < 0.01$; Fig. 3 b) from 24 h to 6 days post-ischemia.

Consistent with these findings, postoperative neurological outcomes on the inclined plane test revealed that ischemic animals presented deficits in climbing at 6 h post-surgery (48.5 inclination grades (ig°)) for the ischemic group and 48.25 ig for rats treated with Linalool compared with the control groups (53.2 ig° for the sham group and 53.47 ig° for the Linalool group). Meanwhile, at 24 h post-ischemia, significant differences were observed between the ischemic group (52 ig°) and the treated group (55 ig°), which was similar to the sham groups (55-57 ig°). These differences persisted for 7 days, when treatment with Linalool significantly improved the climbing skills of the ischemic group (59 ig°) compared to untreated ischemic group (56 ig°) (*$p < 0.05$) (Fig. 3 c). Those findings were supported by the motor performance (latency, distance, and velocity) on the Rotarod test, in which ischemic animals treated with Linalool exhibited better performance on day 4 than the ischemic group (*$p < 0.05$) (Fig. 3 d-f). However, the already higher motor skills (latency, distance, and velocity) of the sham group also improved 5e7 days after treatment with Linalool compared to the sham vehicle group (*$p < 0.05$) (Fig. 3 d-f).

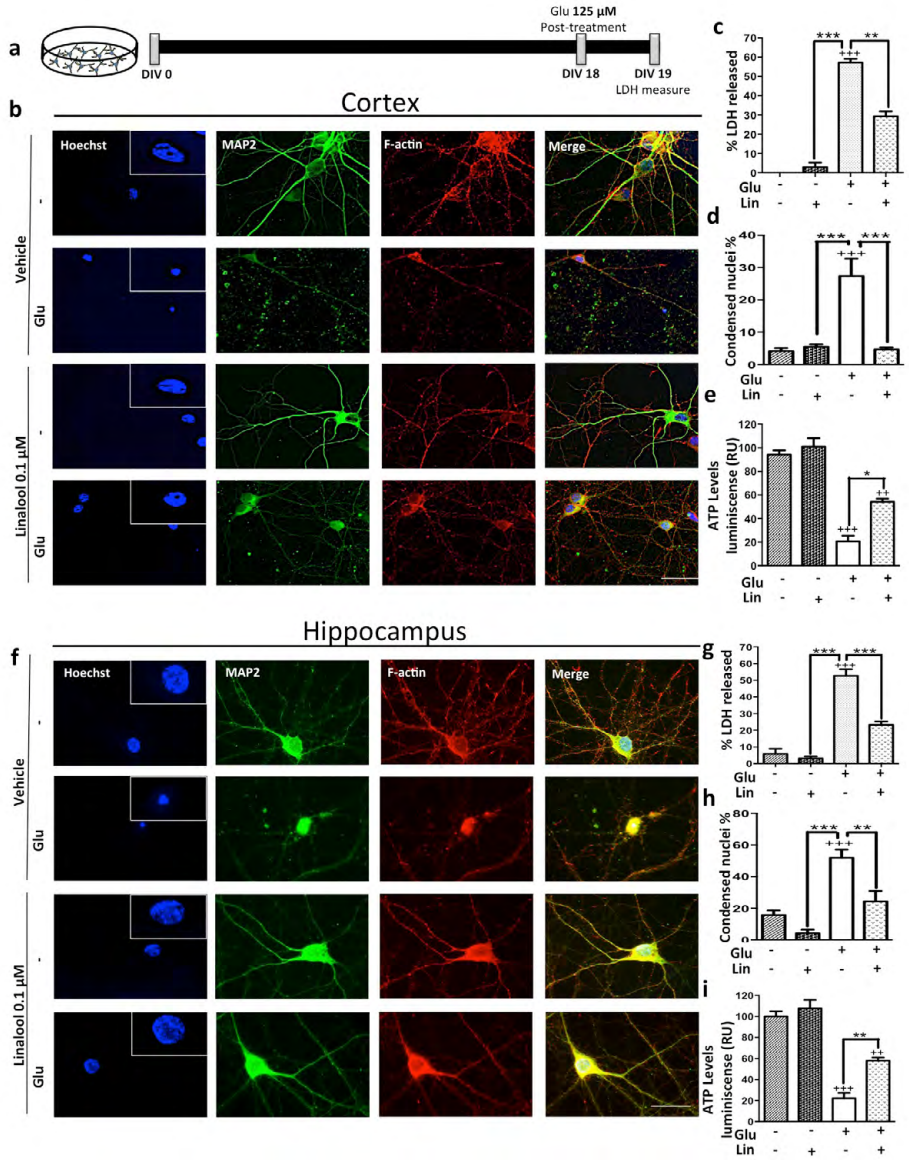


Fig. 1. Responses of neuronal cultures to glutamate excitotoxicity and treatment with Linalool. (a) Scheme of the experimental design; Morphological characterization of mature neurons post-treated with 0.1 µM Linalool from cortices (bee) and hippocampi (f-i) respectively. The nuclei were stained with Hoechst (blue), the F-actin cytoskeleton was stained with an Alexa Fluor 594-conjugated phalloidin dye (red) and dendrites were stained with MAP2 visualized with an Alexa Fluor 488 dye (green). Magnification: 40x, scale bar: 20 µm. (c, g) Neuronal cytotoxicity is presented as the percentage of LDH released from the cells 24 h after glutamate exposure by mature neurons. (d, h) Ratio of condensed nuclei to total nuclei for each treatment (shown as %). Condensed nuclei were quantified from 450 Hoechst-positive cells for each treatment. (e, i) ATP levels were determined using a luminescent cell viability assay. Data are presented as the means ± SEM of three experiments performed in duplicate. $^{+}p < 0.05$ ANOVA with Tukey's test compared to the neurons that were not treated with glutamate (control). *p < 0.05 and **, p < 0.01, ANOVA with Tukey's tests compared to neurons treated with glutamate.

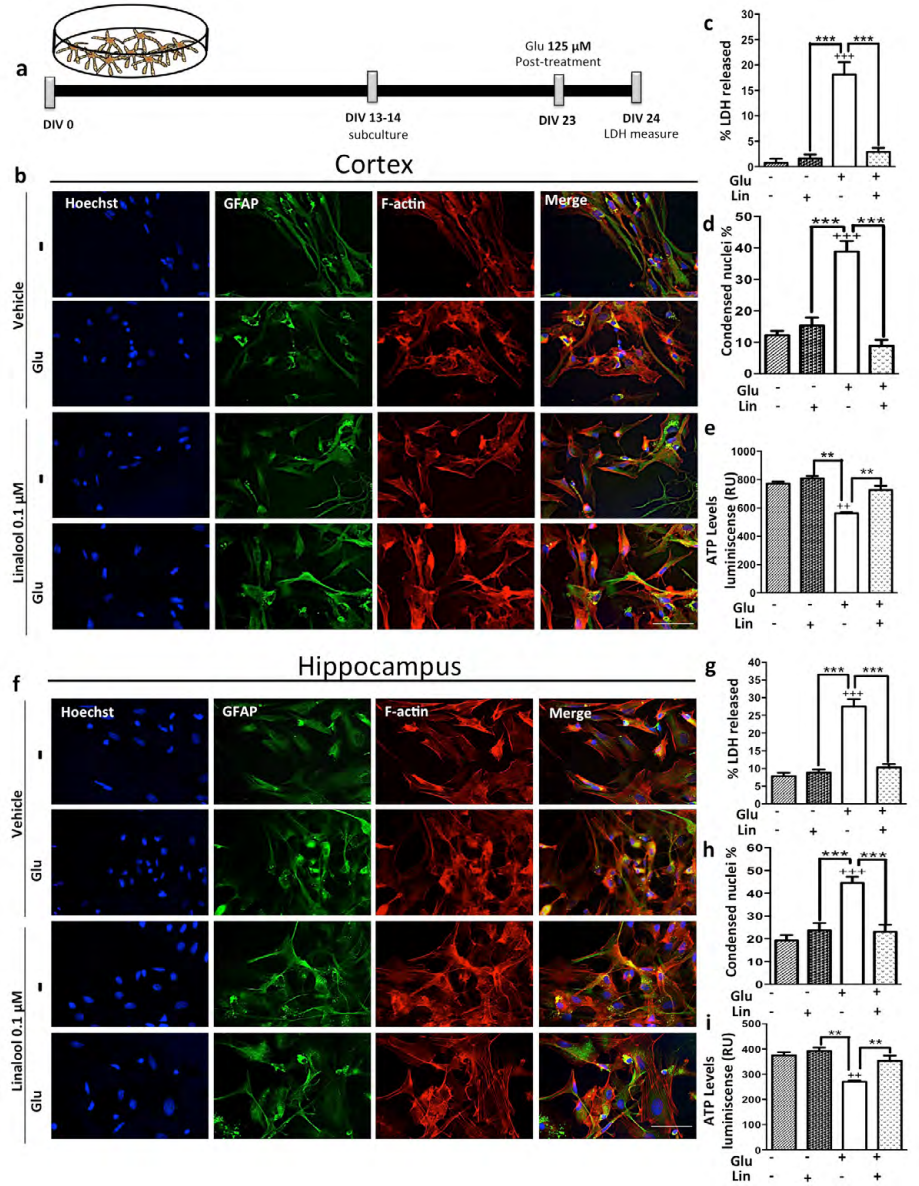


Fig. 2. Astrocytes response glutamate-mediated toxicity and Linalool treatment. (a) Scheme of the experimental design; astrocytes from cortices (bee) and hippocampi (f-i) were treated with glutamate at DIV 23 for 20 min and post-treated with 0.1 µM Linalool for 24 h (b, f) Morphological characterization of the nuclei stained with Hoechst (blue), F-actin cytoskeleton stained with Alexa Fluor 594-conjugated Phalloidin dye (red) and astrocytes stained with GFAP visualized with an Alexa Fluor 488 dye (green). Magnification: 40 x, scale bar: 20 µm. (c, g) Astroglial cytotoxicity is presented as the percentage of LDH released from the cells 24 h after glutamate exposure. (d, h) Ratio of condensed nuclei to total nuclei for each treatment (shown as %). Condensed nuclei were quantified from 450 Hoechst-positive cells for each treatment. (e,i) ATP levels were determined using a luminescent cell viability assay. Values are presented as the means ± the SEMs of n: 3 experiments, and each experiment was performed in duplicate. $^{+}p < 0.05$ ANOVA with Tukey's test compared to the astrocytes that were not treated with glutamate (control). *p < 0.05 and **, p < 0.01, ANOVA with Tukey's tests compared to the astrocytes treated with glutamate.

In the spatial navigation test, cognitive performance was assessed by determination of the animal's escape latency in learning and re-learning trials. The analysis of the escape latency revealed significant differences among training days, particularly between trials 3, 4 and 6 (***p < 0.001), where the ischemic group treated with Linalool exhibited better performance than the ischemic group and a similar performance to the control groups (Fig. 3 e, *p < 0.05). Forty-eight hours after the last training trial, the platform was removed from the maze. In the retention test, rats treated with Linalool exhibited lower latencies to find the platform site than the ischemic group (**p < 0.01) and similar performance to

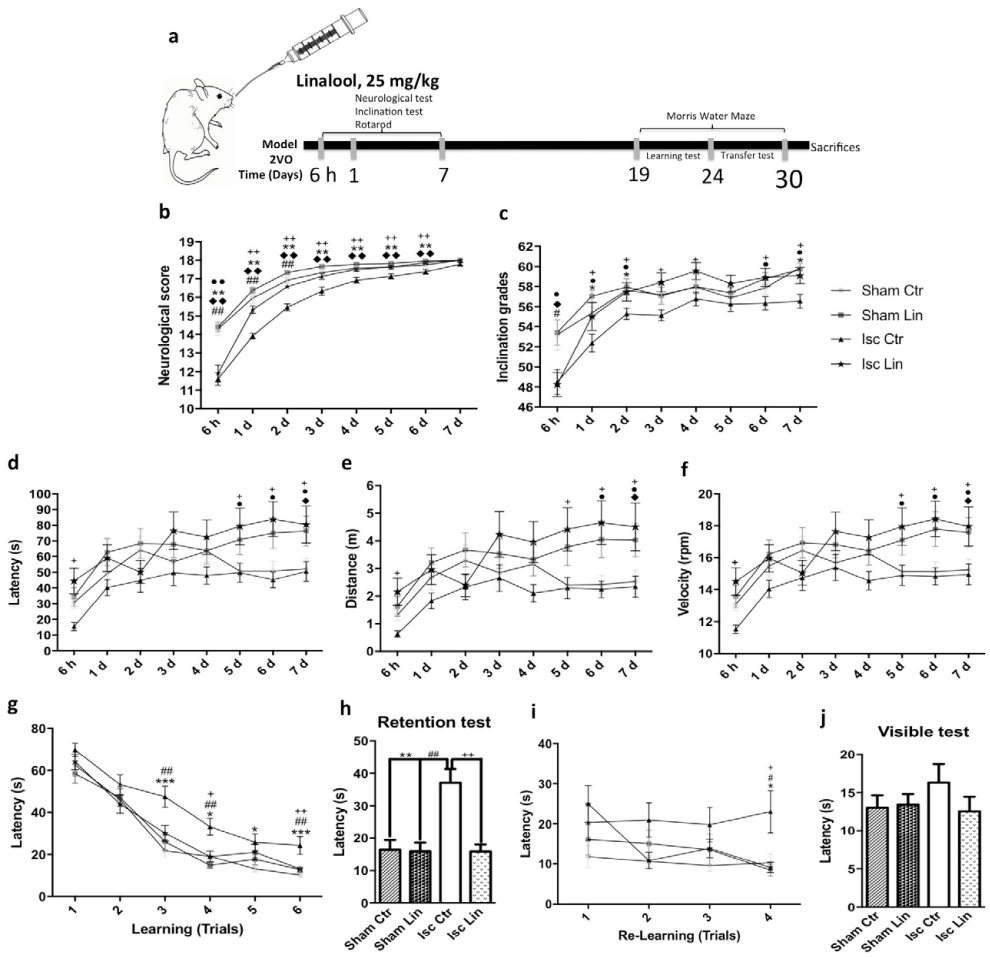


Fig. 3. Neurological, motor and cognitive skills in ischemic rats treated with oral Linalool. (a) Scheme of the design of the *in vivo* experiment, where the behavioral tests began 6 h after the global ischemia or sham operations. Consolidated data were recorded from the behavioral tests at 6 h after the operation and then every 24 h for 7 days. (b) Neurological score. (c) Incline plane test. (d) Latency, (e) distance and (f) velocity in the Rotarod test. (g) The Morris water maze test was evaluated on day 19, starting with learning test (first position of the platform). (h) Retention test after 48 h without test. (i) Transference test (second position of the platform). (j) Visible test. Data are expressed as group means ± SEM. *p < 0.05, **p < 0.01; ***p < 0.001. *sham + Ctr vs isc + Ctr; #sham + Lin vs isc + Ctr, +Isc Ctr vs isc Lin, ♦sham + Lin vs isq + Lin; •sham Ctr vs isq Lin. n: 20- 24 animals/group.

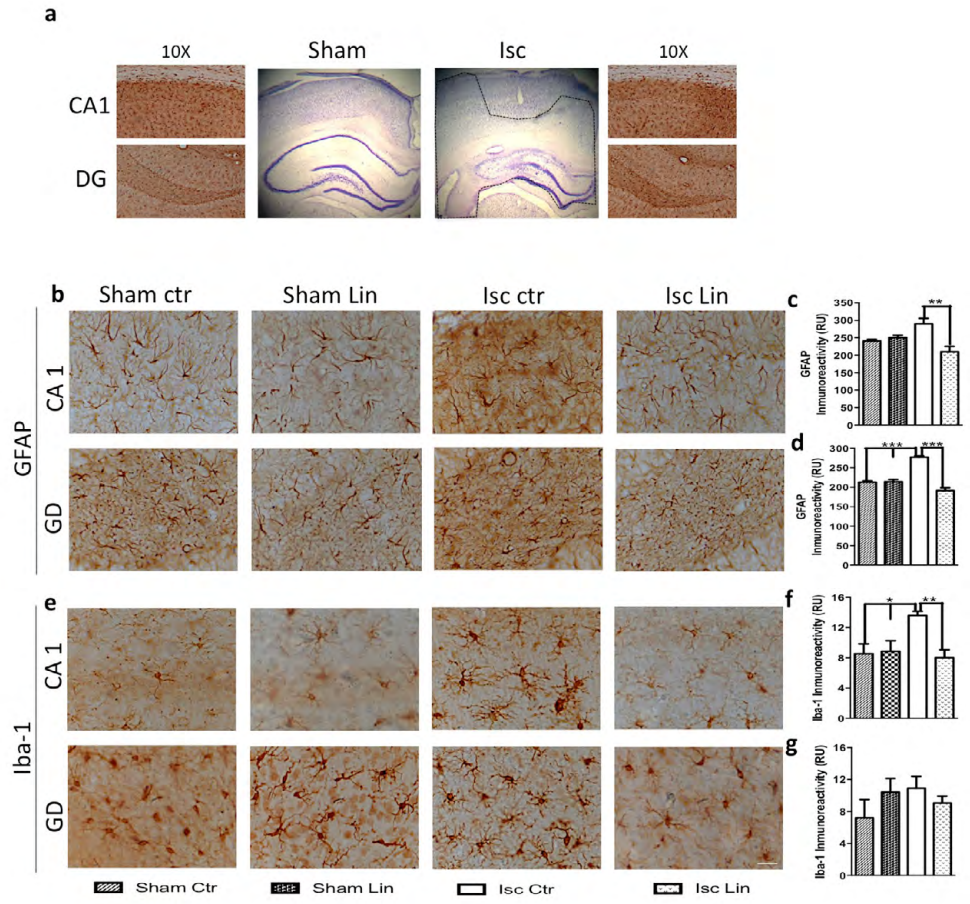


Fig. 4. Linalool reduces astrogliosis in the hippocampus at one month post-ischemia. (a) Representative panoramic images of Nissl staining from sham and ischemic rats. Infarcted area is highlighted f by dotted borderline. Panoramic micrographs of GFAP immunoreactivity are showed from the ipsilateral CA1 area and DG of the hippocampus in both conditions. Magnification: 10x. (b) GFAP immunoreactivity of the ipsilateral CA1 area and DG of the hippocampus. 40x magnification. (c) Values in the bar graphs are expressed as relative densitometric units (RU) of GFAP immunoreactivity in the CA1 area and (d) DG. Representative images of Iba-1 immunoreactivity in the CA1 area and DG of the hippocampus. Values in the bar graphs are expressed as relative densitometric units (RU) of Iba-1 immunoreactivity in (e) the CA1 area and (f) DG. Control: vehicle (PEG); Lin: Linalool; DG: dentate gyrus. Data are expressed as means ± SEM. n :4-5 per group. *p < 0.05 **p 0.01; ***p < 0.001. Scale bar :50 µm.

control animals (Fig. 3 f). In addition, during the re-learning test, ischemic rats treated with Linalool exhibited an improved latency in trial 4 compared with the untreated ischemic group (Fig. 3 g). The visible test did not reveal any visual, motor or motivational deficits in the experimental groups (Fig. 3 h).

3.3. Post-ischemic pro-inflammatory response was prevented by the oral Linalool treatment

Once the behavioral tests were complete, animals were euthanized, and their brain tissues were assessed using histology, showing by Nissl affected brain zones by the global

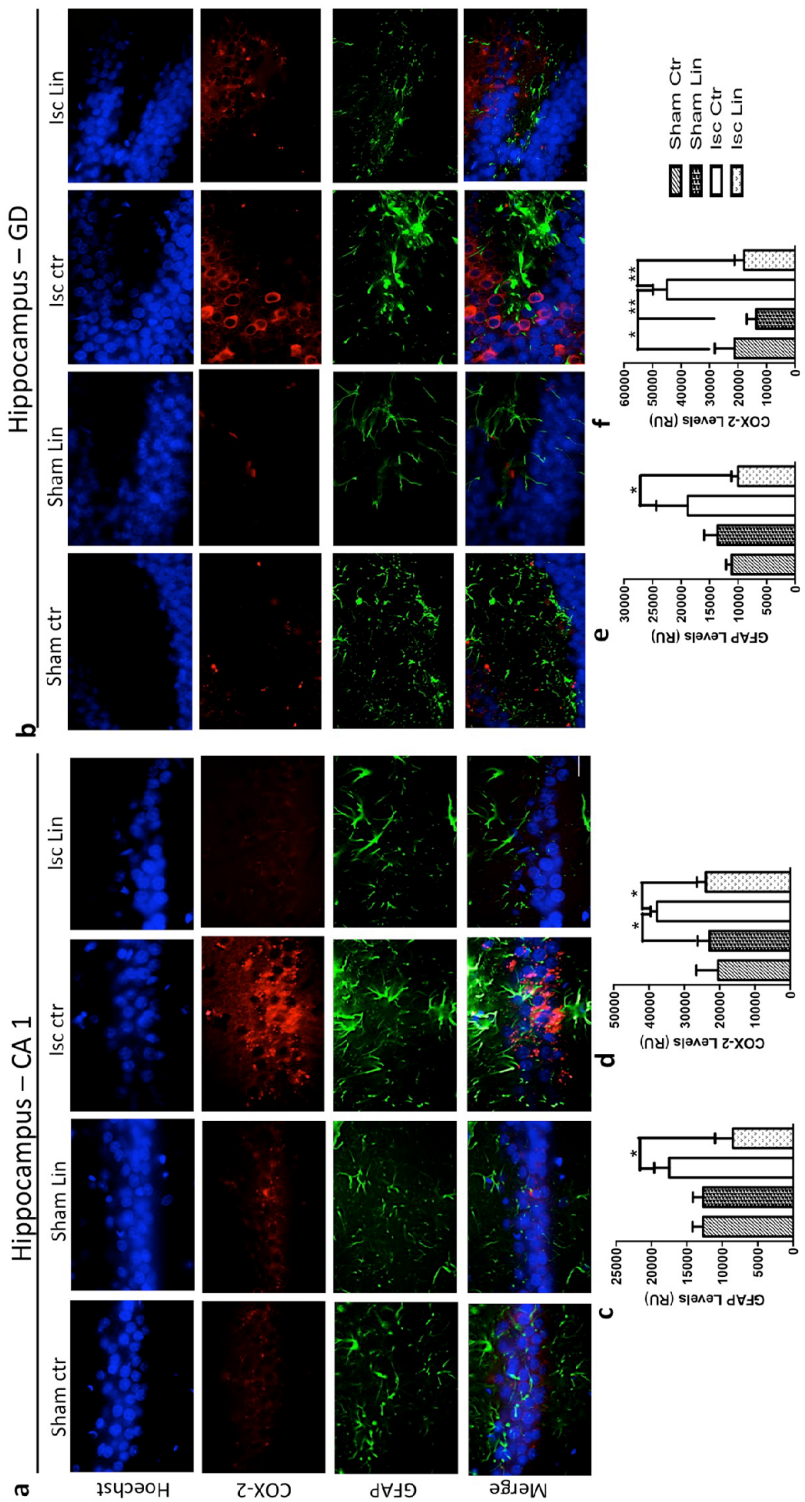


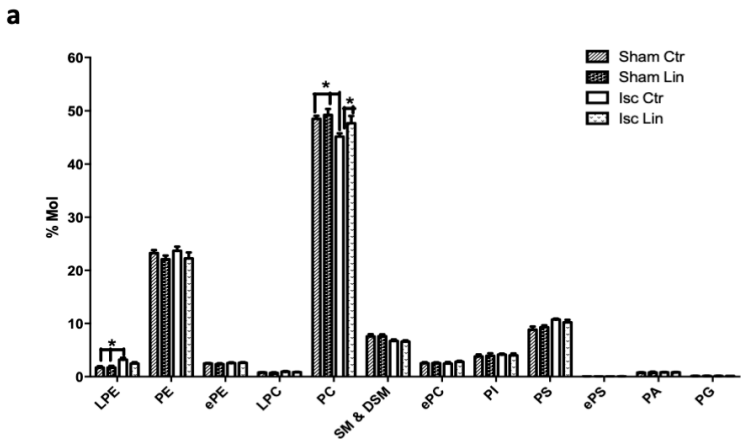
Fig. 5. Post-ischemic pro-inflammatory response was prevented by oral Linalool. Morphological characterization showing nuclei stained with Hoechst (blue), COX-2 staining labeled with the Alexa Fluor 594 dye (red) and astrocytes visualized with Alexa Fluor 488-conjugated to GFAP (green). Magnification: 60x. (a) Representative images of the CA1 area and (b) DG of the hippocampus. (c) Values in the bar graphs are expressed as relative densitometric units (RU) of GFAP immunoreactivity and (d) COX-2 protein levels in the CA1 region and (e-f) the DG. Control: vehicle (PEG); Lin: Linalool; DG: dentate gyrus. Data are expressed as means ± SEM. n: 4-5 per group. *p < 0.05 **p 0.01; ***p < 0.001.

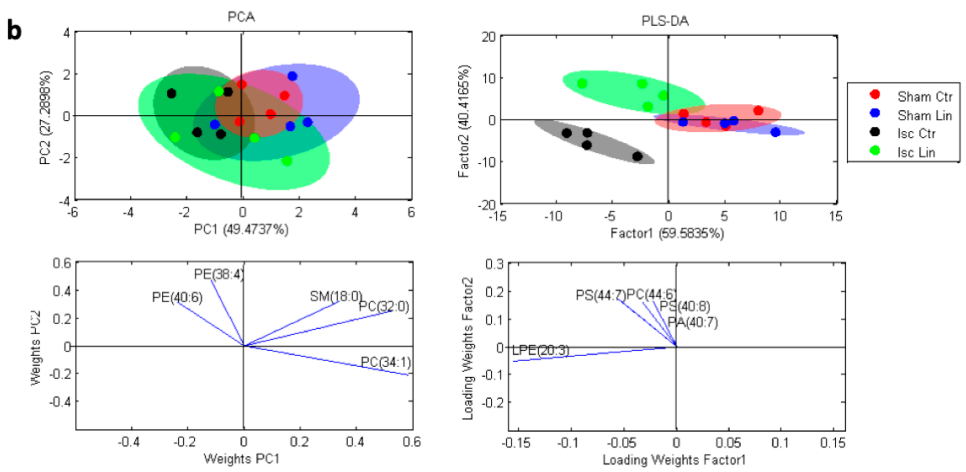
ischemia (Fig. 4 a). We evaluated the neuronal populations between different experimental groups, but no changes were observed (data not shown). However, GFAP-immunoreactive astrocytes in the hippocampal CA1 and dentate gyrus (DG) (Fig. 4 a) showed morphological changes, such as hypertrophic astrocytes with thickened processes at 40x (Fig. 4 b), which displayed a substantial increase in immunoreactivity one month after global ischemia compared with sham groups (Fig. 4 c, 4 d). Surprisingly, the administration of Linalool for one month significantly decreased astrogliosis (*p < 0.05) in the hippocampus (Fig. 4 b-d). Similarly, Iba1- immunoreactive microglia was observed in the same brain areas (Fig. 4 e), presenting considerably increase in the hippocampal CA1 area at day 30 post-ischemia, being significantly reduced by the Linalool treatment (**p < 0.01) (Fig. 4 e, 4 f), although modifications were not observed in the DG (Fig. 4 e, 4 g). Interestingly, we observed the same effect in other areas of the brain as motor cortex (Suppl. Fig. 2) that supports the motor recovery of animals treated with Linalool. These data suggest stressing and pro-inflammatory response induced by global ischemia after one month mainly in the CA1 area, and Linalool prevented the proliferation of microglia and astrocytes at that late phase.

We verified the inflammatory state using COX-2 staining. COX-2 immunofluorescence presented neuronal location, which was significantly increased in the CA1 and DG areas of ischemic rats at one month post-stroke (Fig. 5 a, 5 b), and these changes were markedly reduced by the Linalool treatment (p < 0.05) (Fig. 5 d, and 5 f), accompanied by the significant reduction in astrogliosis shown by GFAP immunostaining (Fig. 5 c, 5 e). Briefly, Linalool attenuated ischemia-induced COX-2 up-regulation, and ischemia-induced astrocyte and microglial activation in the hippocampal CA1 region. Nevertheless, changes in survival and inflammation-related signaling were not detected in the hippocampus at one month post-ischemia (data not shown).

3.4. Alterations in the hippocampal phospholipid profile in ischemic rats were attenuated by Linalool

Three hundred eleven species of phospholipids were evaluated by mass spectrometry to understand the effects of one month of ischemia on the hippocampal phospholipid profile. At a glance, the analysis revealed two types of variations in the phospholipid contents. The first set of changes seemed to be related to the pathological condition, whereas the second type is associated with the Linalool treatment. The lipid profile of the hippocampus in the sham and ischemic groups showed a primary composition of highly abundant glycerophospholipids, such as PC (48.8 and 47.7%), PE (22.6 and 22.8%), PS (9.0 and 9.7%), and PI (3.8 and 3.9%); sphingolipids, such as SM-DSM (7.6 and 7.1%); low abundance ether phospholipids, such as ePC (2.51 and 2.56%), ePE (2.41 and 2.48%), and ePS (0.02 and 0.03%); lysophospholipids, such as LPE (0.2 and 0.4%) and LPC (0.73 and 0.79%) and glycerophospholipids, such as PA (0.75 and 0.77%) and PG (0.12 and 0.12%), respectively (Fig. 6 a).





PCA	Rho
PC(34:1)	1.6040
PC(32:0)	1.5264
SM(18:0)	1.1733
PE(40:6)	0.9523
PE(38:4)	0.8739

PLS-DA	VIP
PC(44:6)	2.1507
PS(44:7)	2.0674
PS(40:8)	1.9796
PA(40:7)	1.9252
LPE(20:3)	1.9094

Fig. 6. Phospholipid composition of the hippocampus from ischemic rats treated with Linalool. (a) The lipid class profiles are expressed as % mol composition. All lipid species are represented as the means ± SEM. The data for ischemic rats were significantly different from the control groups ($p < 0.05$, ANOVA followed by the Tukey post hoc test or Kruskal-Wallis test). Zoom: Changes in a specific PC subspecies. (b) Multivariate analyses of the lipid profiles in the hippocampus. PCA, principal component analyses of the lipid classes; PLS-DA, partial least squares analysis to discriminate between the lipid classes. The left panels illustrate the factor loadings for PC1 and PC2, with the indices of variance explained for each component. The right panels show the factor score plots for PLS-DA. PA, phosphatidic acid; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; ePC ether phosphatidylcholine; PS, phosphatidylserine; ePS, ether phosphatidylserine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; ePE, ether phosphatidylethanolamine, PI, phosphatidylinositol; PG, phosphatidylglycerol; SM, sphingomyelin. n : 4 per group. c) The free fatty acid profile in the hippocampus is expressed as % mol (**p: < 0.01, ***p: < 0.001) ANOVA followed by the Tukey post hoc test or Kruskal-Wallis test. n: 4 per group.

The Linalool treatment resulted in small changes in the lipid profile of ischemic rats; ischemia induced a significant decrease in the PC content (control vs ischemic vehicle, $*p < 0.05$) that was restored by Linalool ($*p < 0.05$) (Fig. 6 a), suggesting a regulatory role of the substance in the biosynthesis of this phospholipid. In addition, we observed increases in the LPE contents in general and specifically in the LPC 22:6 content in the ischemic group compared with the sham group ($*p < 0.05$), but no significant differences were observed after the Linalool treatment, only a slight tendency to reduce these levels (Fig. 6 a and 6 a zoom).

Based on the results of PCA of the detected phospholipids, approximately 76% of the total variance is explained by the first two principal components (PC1 and PC2) (Fig. 6 b). The most relevant variables included in these two components were related to the 34:1 PC subclasses, consistent with the Rho index (1.6). PC 34:1 is composed of saturated palmitic acid (16:0) and oleic acid (18:1). Similarly, the PLS-DA showed similar ellipsoid locations of the control groups and ischemic rats treated with Linalool in a different quadrant to the ischemic group; the more discriminant species was PC 44:6 (20:6/24:0), with a VIP index of 2.15 (Fig. 6 b). Interestingly, the gas chromatography analysis depicted a very specific increase in the lignoceric acid (24:0) content (a peroxisomal failure marker (Singh and Singh, 1986) in the free fatty acid fraction of the ischemic hippocampus ($**p < 0.01$), without modifications in other free fatty acids or in other fractions, such as cholesterol esters and triglycerides (data not shown). These findings could suggest a failure in b-oxidation caused by ischemia, whereas the treatment with Linalool reduces its levels to control values ($***p < 0.001$) (Fig. 6 c).

The ANOVA showed that the ischemic hippocampus displayed significant changes in the pool of two lipid classes: PC and LPE. Next, we evaluated which specific phospholipidic subspecies in those two classes were altered. Then, we focused on PC phospholipids and PCA showed that the subspecies exhibiting the greatest change in abundance was PC 34:1 (Rho index 1.3), which showed a tendency to be decreased in the ischemic group and was a weakly restored by the Linalool treatment (Fig. 7 a-c). Nevertheless, the PLS-DA presented more separability (Fig. 7 a), showing that the ischemic group exhibited a displacement to the left quadrant, whereas the ischemic Linalool-treated group occupied a different region that was shifted to the right, closer to the sham groups (Fig. 7a). Other PC subspecies were less abundant, but the PC 34:2 content was significantly reduced ($**p < 0.01$), presenting an opposite pattern to PC 42:1 (24:0/18:1), which was increased ($*p < 0.05$) in the ischemic group, compared with both the ischemic Linalool treated group and sham groups (Fig. 7 d).

Regarding LPE, the PCA and PLS-DA revealed that ischemic group was almost completely separated from the other experimental groups (Fig. 8 a). LPE 18:1, 20:3 and 22:6 levels were significantly increased in the ischemic group ($*p < 0.05$) (Fig. 8 b), and these changes were partially reversed by the Linalool treatment in ischemic rats at one month post-stroke (Fig. 8 c).

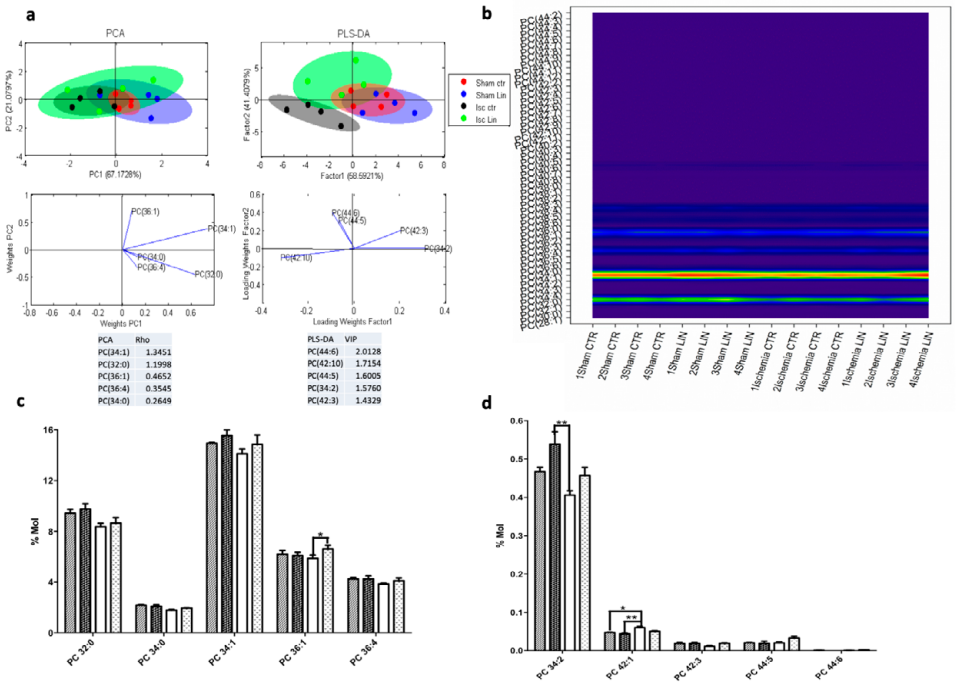


Fig. 7. Linalool partially restores the basal levels of PC at one month post-ischemia. (a) PCA, principal component analyses for the PC subclasses, and PLS-DA, partial least squares method to discriminate between the PC subclasses. The left panels illustrate the factor loadings for PC1 and PC2, with the indices of variance explained for each component. The right panels show the factor score plots for PLS-DA. (b) Contour plot of the more influential subclasses of PC (variables) in the discriminant analyses of each evaluated variable. (ced). The lipid class profiles are expressed as % mol PC composition. (*p:< 0.05, **p:< 0.01 ANOVA followed by the Tukey post hoc test or Kruskal-Wallis test); all PC subclasses were measured (means), and the error bars represent the SEM. PC: phosphatidylcholine. n :4 per group.

3.5 Pro-inflammatory phosphatidylinositol composition was increased in the serum of ischemic rats and was attenuated by the oral Linalool treatment

Finally, we evaluated the phospholipid profile in serum and observed changes in specific subspecies of the PI class (Fig. 9). Although the PCA did not show differences among experimental groups, the PLS-DA exhibited a differential displacement of the ischemic group compared to the other groups, with the ischemic Linalool-treated group located in the right quadrant close to the sham groups (Fig. 9 a). Increases in PI 36: 2 (18:1/18:1), PI 38: 4 (18:0/20:4) and PI 38:5 (18:1 and 20:4) levels were also observed, with a higher abundance of PI 38:4 (18:0/20:4), as shown in the counter plot analysis (Fig. 9 b and c). These findings suggest that PI includes arachidonic acid, a typical pro-inflammatory fatty acid. The Linalool treatment significantly decreased the levels of those phospholipid subspecies, suggesting that a reduction in peripheral inflammation signaling from the brain may be attenuated by the monoterpene Linalool at one month post-ischemia.

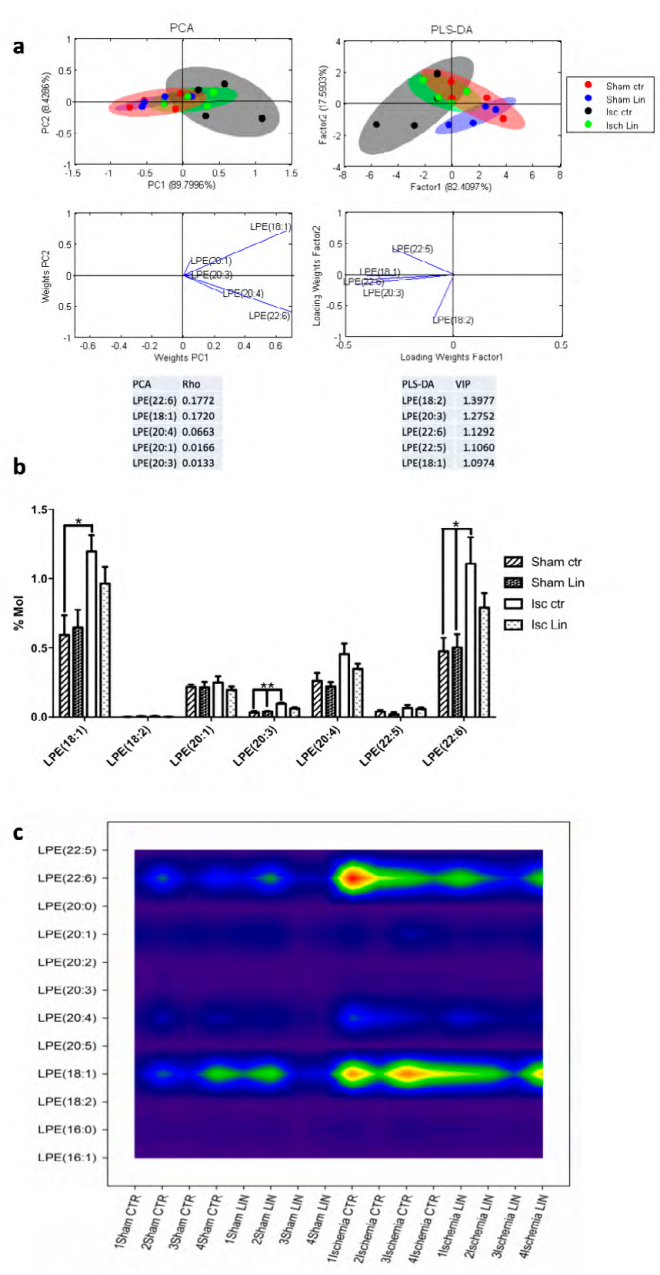


Fig. 8. Changes in LPE levels in ischemic animals and effects of Linalool at one month post-ischemia. (a) PCA, principal component analyses for the LPE subclasses, and PLS-DA, partial least squares method to discriminate between the LPE subclasses. The left panels illustrate the factor loadings for PC1 and PC2, with the indices of variance explained for each component. The right panels show the factor score plots for PLS-DA. (b) The lipid class profiles are expressed as % mol LPE composition (p: < 0.05, p: < 0.01 ANOVA followed by the Tukey post hoc test or Kruskal-Wallis test). (c) Contour plots of the more influential subclasses of LPE (variables) in the discriminant analyses of each evaluated variable. All LPE subclasses were measured (means), and the error bars represent the SEM. LPE: lysophosphatidylethanolamine. n : 4 per group.

4. Discussion

Our data are the first to show that cognitively impaired ischemic rats present increased lysophospholipid levels (LPE 18:1, 20:3, 22:6 and LPC 22:6) and a reduction in PC 16:1/18:1 levels in the hippocampus, under a neurodegenerative context, as evidenced by astrogliosis and COX-2+ immunostaining at one month postischemia. The latter was recently shown to be responsible for lysophospholipid oxidation (Liu et al., 2016; Richter et al., 2015). Those neuropathological manifestations were also supported by the substantial discrimination of PC 44:6 (20:6/24:0) and PC:42:1 (18:1/24:0) in ischemic rats respect to the control groups, whose compositions are related to cerebrovascular disease (Davis et al., 2017) and neurological deterioration (Yi et al., 2016); as well as the accumulation of free lignoceric acid (24:0), an indicator of peroxisomal and β-oxidation failure (Singh et al., 1989; Singh and Singh, 1986). In addition, high levels of PI 18:0/20:4, associated to anoxia (Kim et al., 2017) and cerebrovascular events (Toschi et al., 1998), were increased in the serum at long-term post-ischemia period. However, an aromatic monoterpene, Linalool, which is involved in inhibiting cholesterol biosynthesis by reducing levels of HMG-CoA (Rodenak Kladniew et al., 2014); in our study reduced LDH release and avoided ATP depletion in mature neurons and astrocytes from cortex and hippocampus, and reduced lipid peroxidation in mature cortical neurons and astrocytes affected by glutamate excitotoxicity. The effect on lipid peroxidation was not reproduced in mature neurons and astrocytes from hippocampi, maybe related to a differential expression of ionotropic receptors and transporters giving a tissue-specific buffering capacity and stress response. However, Linalool prevented astrogliosis and microgliosis of the motor cortex and the CA1 region, and blocked the hippocampal and peripheral phospholipid profile imbalance, supported by the reduced ischemia-induced degenerative response, consequently producing neurological, motor and cognitive recovery.

Aromatic compounds generally have potent radical scavenging activities and possess the ability to directly interact with lipids and proteins, thereby playing important biological roles (33, 34). Linalool, a major aromatic terpenoid in teas and herbal essential oils, has been traditionally used for medicinal purposes because of its potent antioxidant activities. The oral administration of Linalool has recently been shown to improve dyslipidemia by reducing plasma TG and LDL concentrations (Rodenak Kladniew et al., 2014). Although in our study we did not find changes in other lipid fractions.

On the other side, glutamate plays an important role in many neuronal functions, such as neuronal development, synaptogenesis, neuronal plasticity, learning, and memory processes. However, glutamate-induced excitotoxicity has also been suggested to underlie the pathogenesis of neuronal damage and degeneration following *in vivo* cerebral ischemia and *in vitro* oxygen deprivation (Parpura et al., 2016). Our data revealed the protective effect of Linalool on glutamate-induced excitotoxicity in neurons and astrocytes, which

Changes in the hippocampal and peripheral phospholipid profiles are associated with neurodegeneration hallmarks in a long-term global cerebral ischemia model: Attenuation by Linalool

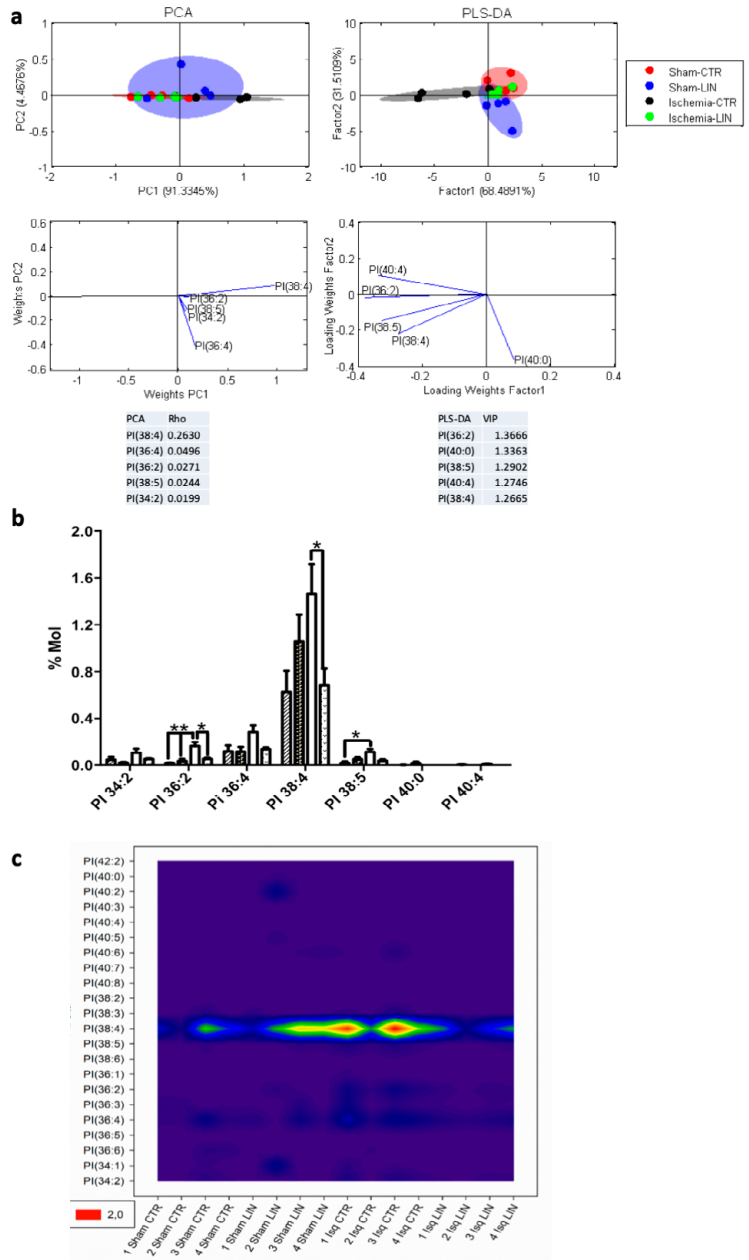


Fig. 9. PI profile in the serum of ischemic rats and rats treated with Linalool for a month. (a) PCA, principal component analyses for the PI subclasses, and PLS-DA, partial least squares method to discriminate between the PI subclasses. The left panels illustrate the factor loadings for PC1 and PC2, with the indices of variance explained for each component. The right panels show the factor score plots for PLS-DA. (b) The lipid class profiles are expressed as % mol PI composition (p: < 0.05, p: < 0.01 ANOVA followed by the Tukey post hoc test or Kruskal-Wallis test). (c) Contour plots of the more influential subclasses of PI (variables) in the discriminant analyses of each evaluated variable. All PI subclasses were measured (means), and the error bars represent the SEM. PI: phosphatidylinositol. n: 4 per group.

were supported by previous *in vitro* studies in which PC12 cells were cultivated for 8 h in GSD (glucose/serum deprivation) conditions. In the presence of Linalool (16 mg/ml), the cell viability increased to 45% (Alinejad et al., 2013). Another recent study assessed the neuroprotective effects of Linalool against oxygen-glucose deprivation/reoxygenation (OGD/R)-induced cortical neuronal injury, where Linalool significantly reduced intracellular oxidative stress, scavenged peroxy radicals, and decreased the activities of SOD (superoxide dismutase) and catalase and microglial activation (Park et al., 2016).

The present study is the first to show the robust recovery of neurological, motor and cognitive functions in a rat model of global ischemia over the entire testing period of 30 days following treatment with 25 mg/kg/d Linalool. Significant improvements in the neurological score, motor function (inclined plane and rotarod test) and cognitive function (MWM) were observed. We did not find similar studies in the literature. Cognitive impairment is one of the most typical characteristics of subjects with cerebral ischemia. The Linalool treatment effectively decreased the escape latencies in the learning, retention and re-learning tests compared with the ischemic group treated with vehicle. Our group observed analogous results in an aged triple transgenic model of AD, where Linalool prevented spatial memory impairments and reduced the main histopathological markers of AD (Sabogal-Guáqueta et al., 2016). Recently, Xu et al. corroborated this effect in another model of AD, where 100 mg/kg Linalool reduced the escape latencies, increased the escape rate, and increased the time spent exploring the previous platform location in MWM test (Xu et al., 2017).

The cellular and molecular cascades underlying ischemic injury are multifaceted and complex. Excitotoxicity, oxidative stress, and an excessive inflammatory response have been implicated in the progressive neuronal injury and cell death observed post-ischemia (Bhuiyan et al., 2011). Microglia represent the first line of defense against brain injury, and astrocytes are the most abundant cells in the central nervous system, providing structural and nutritional support to neurons. However, in cerebral ischemia, the activation of microglia and astrocytes may induce the production of a variety of cytokines, chemokines and ROS that contribute to enlarging the infarct (Xian et al., 2016). In our study, GFAP and Iba-1 immunoreactivities were reduced in ischemic rats treated with Linalool, which indicates neuroprotection. These results were supported by the *in vitro* findings showing that Linalool also exerts a similar effect on mature astrocytes and neurons reducing condensed nuclei and ATP failure. In a previous study, Linalool treatment of LPS stimulated BV2 microglia cells inhibited the production of TNF-α, IL-1ß, NO, PGE2, and NF-kB in a dose-dependent manner (Li et al., 2015). On the other hand, according to *in vivo* studies, Linalool reduces nociceptive behavior in response to the direct administration of inflammatory mediators in a mouse model of neuropathic hypersensitivity (Batista et al., 2010).

Changes in the hippocampal and peripheral phospholipid profiles are associated with neurodegeneration hallmarks in a long-term global cerebral ischemia model: Attenuation by Linalool

We also evaluated COX-2 expression in the hippocampus. This enzyme is very important for normal brain function and, under pathological conditions, it produces high levels of prostaglandins that generate harmful effects on neural membranes and nuclei (Farooqui, 2009). Ischemic injury is accompanied by increased COX- 2 immunoreactivity in neurons and glial cells (Tomimoto et al., 2000). We observed an increase in COX-2 immunostaining mainly in hippocampal neurons from animals treated with vehicle one month post-ischemia, whereas Linalool reduced the COX-2 immunoreactivity to levels similar to controls. In previous studies in our lab, we observed a reduction in the levels of inflammatory markers, such IL-1ß, p38 MAPK, iNOS and COX-2, in aged 3xTg-AD mice following treatment with Linalool (Sabogal-Guáqueta et al., 2016). Reduced levels of cyclooxygenases have been observed in other neurotoxicity models (Li et al., 2014; Peana et al., 2006).

Complementarily, in this study we analyzed 12 lipid classes that covered over 311 lipid subclasses and detected specific changes in three phospholipids (PL) and four lysophospholipids (LPL) in the hippocampus and three PLs in the serum of rats at one-month postischemia. The ischemic group exhibited decreased levels of PC and increased levels of LPE and LPC, whereas Linalool restored the amounts of these plasmalogens to basal levels that were similar to sham animals, which may be associated with tissue homeostasis and supports the cognitive recovery observed in the MWM test. These results suggest an imbalance between PLs and LPLs that potentially contributes to our understanding of the mechanism involved in cerebrovascular diseases and its potential protective targets.

Phospholipid compositions are complex and involved in maintaining lipid asymmetry, a dynamic process required to maintain normal neural membrane functions, such as neuroplasticity and vesicular transport (Yamaji-Hasegawa and Tsujimoto, 2006). The disruption of asymmetry may lead to the apoptotic cell death observed in cerebral ischemia (Farooqui, 2011) by inducing an energy deficiency and over-stimulation of glutamate receptors, elevated intracellular calcium concentrations and activate phospholipases (PLA 2, C, and D). Activation of these phospholipases causes hydrolysis of membrane phospholipids and the release of second messengers involved in the inflammatory response, maximizing the brain injury (Adibhatla and Hatcher, 2007; Wang and Shuaib, 2002).

An imbalance in the production of PC and LPC was described in early studies on ischemia (Koizumi et al., 2010), which was confirmed by the decreased levels of PC (34:2, 36:1) and increased levels of LPC (22:6) in the ischemic group in our study. Phosphatidylcholine (PC) is the most abundant glycerophospholipid in the membrane and plays a key role in cellular signaling. The balance between synthesis and degradation is controlled by various enzymes, such as PLA_2, PLD, PLC and lysophospholipid acyltransferases (Mateos et al., 2010). Under pathological conditions, such as ischemia, the activities of these enzymes change, resulting in the loss of homeostasis. Previous studies of neurodegenerative diseases have reported

reduced PC and PE levels in the cerebral cortex of patients with AD, which is potentially linked to the roles of PLA_2 a PLD in Ab activation (Blusztajn et al., 2017; Whiley et al., 2014). Reduced PC (16:0/18:1) levels were observed in a focal ischemia model at 24 h (Koizumi et al., 2010). However, in 2016, Miyawaki et al. observed that an increase in PC (diacyl-16:0/18:1) levels in the hippocampus 21 days after global ischemia was induced (Miyawaki et al., 2016). Based on these results, the PC distribution patterns depend on the fatty acid composition, which reflects the heterogeneous membrane lipid compositions in distinct cell types. In addition, PC species are composed of saturated palmitic (16:0) and stearic acid (18:0), monounsaturated oleic acid (18:1) and polyunsaturated linoleic acid (18:2), and this composition of membrane lipids determines their fluidity, curvature and permeability. For example, linoleic acid-containing PC species are reduced during the apoptotic process in ischemia (Monteiro et al., 2013). Our results confirmed the imbalance in PC biosynthesis after global cerebral ischemia and suggest the recovery of the homeostasis of those phospholipids following treatment with Linalool.

In addition, we observed an increase in LPE levels in the hippocampus of ischemic groups. LPE is generated from PE via phospholipase, and it has been reported to be synthesized in rat brain synaptosomes and is markedly stimulated by calcium (Farooqui, 2011). Specifically, we showed an increase in levels of LPE 18:1 (oleic acid), 20:3 eicosatrienoic acid (ETE) and 22:6 (DHA) FA chains. Previous studies of cerebral ischemia have reported increased levels of oleic acid and DHA in the ischemic region compared with the contralateral region (Baskaya et al.,1996; Pilitsis et al., 2002). As shown in a recent study by our group, LPE levels are increased in the hippocampus of AD triple transgenic animal model, which are also composed of DHA and oleic acid and are strongly correlated with the pro-inflammatory response (Villamil- Ortiz et al., 2016). Furthermore, the PLS-DA of the phospholipid species suggested that the ischemic group has a different pattern from the ischemic Linalool-treated group and sham groups. Another lipid subspecies that may explain this difference is LPE (20:3), a polyunsaturated fatty acid that has not been described in ischemia, but it has been described to be more vulnerable to oxidation because it contains double bonds that are each separated by one methylene group. The esters are readily oxidized by free radical-mediated chain oxidation, promoting lipid peroxidation that is involved in the impairment of membrane, proteins and enzymes in various diseases (Niki, 2014).

On the other hand, lysophospholipids (LPLs) are proinflammatory phospholipids that are synthesized in the brain through the action of PLA 1 and PLA 2 on phospholipids, such as PC and PE, which are metabolized by lysophospholipases and acyltransferases. LPLs, such as LPC, not only directly interact with ion channels and neurotransmitter receptors but also indirectly modulate their activity and neural membrane fluidity (Farooqui and Horrocks, 2007). LPC 22:6 levels were increased in the ischemic group compared to the other groups. LPLs such as LPC and LPE alter the membrane permeability and disturb the osmotic

equilibrium. In addition, the reduction in PC levels suggests the enzymatic deregulation between LPLs and PLs at one month postischemia. However, we did not observe changes in the $cPLA_2$ protein levels, suggesting that the other isoforms of this enzyme are involved. Moreover, a neuronal population-specific regulatory mechanism may exist, whose changes may be diluted in the hippocampal lysates or a regulatory mechanism functions before the evaluation at one month post-ischemia; thus, more studies are needed to clarify these hypotheses.

Among the families of lipids examined in the present study, the phosphatidylinositol (PI) family was present in low percentages. However, we detected increased levels of PI 36:2, 38:4 and 38:5 in the serum of ischemic animals. Interestingly, the Linalool treatment reversed these levels to values similar to controls. PI is a phosphoinositide located in the inner side of the membrane and a majority of its biological functions focus on phosphorylation of its inositol head group that regulate many fundamental processes in the cell, including membrane trafficking, cell growth, cytoskeletal remodeling and nuclear events. These regulatory actions are mainly due to the ability of these lipids to control the subcellular location and activation of various effector proteins that possess PI-binding domains and are involved in vesicle trafficking, such as the PH, FYVE, PX, ENTH, PH-GRAM, FERM and GLUE domains (Sasaki et al., 2007; Vicinanza et al., 2008). Also, PI response to calcium stimuli in the brain (69), its imbalance is associated to mitochondrial dysfunction (70) and to a toxic environment in neurological diseases (71), and this phospholipid has been associated to vascular alterations (72); maybe suggesting a peripheral biomarker of neurovascular unit damage progression, but it is necessary a deeper analysis.

Finally, an additional novel finding is the property of Linalool to restore the phospholipid profile, specifically the hippocampal PC, LPC and LPE levels and peripheral levels of PI subspecies in ischemic rats. Linalool also induced better performance of ischemic rats on motor and cognitive tests at one month post-ischemia. No previous studies have reported these types of effects, although Linalool has been shown to inhibit steps in cholesterol synthesis by blocking hydroxy-methyl-glutaryl-coenzyme-A reductase (HMGCR) levels (Rodenak Kladniew et al., 2014). Similarly, oral administration of Linalool to mice for 6 weeks significantly reduced total and lowdensity lipoprotein cholesterol concentrations and the levels of the HMG-CoA reductase protein (Cho et al., 2011). Moreover, Linalool stimulation reduced cellular lipid accumulation, regulated PPARα-responsive genes (32), and reduced the oxidation of unsaturated FAs (Celik and Ozkaya, 2002; Jun et al., 2014), which supports our observations, but detailed future studies must be done.

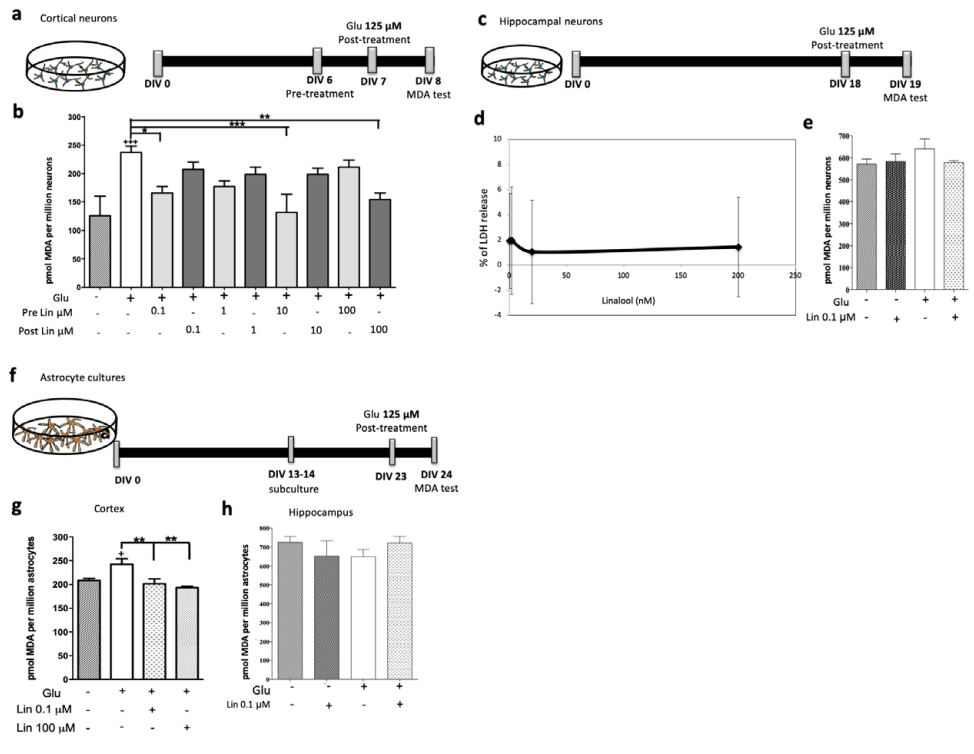
5. Conclusion

In summary, neurodegeneration produced by excitotoxicity in subjects with cerebral ischemia is still observed one month postinjury and is part of the neurological disorders and cognitive dysfunction pathogenesis, as supported by the PC deficiency and astrogliosis environment in the hippocampus, accompanied by increased levels of lysophospholipids, such as LPE and LPC composed by PUFAs with inflammatory properties, and reflected in the periphery by the release of PI composed of stearic and arachidonic acids (18:0/20:4). Interestingly, the monoterpene Linalool reversed accumulation of PUFAs and plasmalogens potentially through its action on peroxisomal oxidation, because it had a very specific effect on free lignoceric acid levels in the hippocampus, since other lipid fractions or signaling cascades were not modified by the oral therapy at one month post-ischemia. Nevertheless, additional studies are necessary to confirm those lysophospholipids as biomarkers of neurological and cognitive impairment progression after ischemia and to define the specific mechanism of action of the monoterpene as a potential therapeutic substance after stroke.

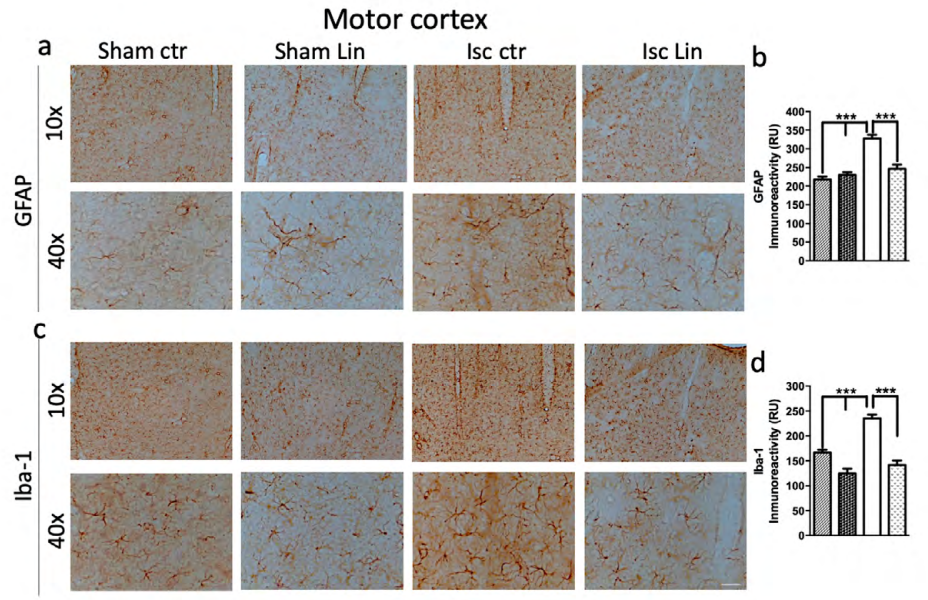
Acknowledgments

The authors thank the Cellular and Molecular Neurobiology Area of the Neuroscience Group of Antioquia, the Group of Bioactive Substances, Professor Marisol Lamprea and Gonzalo Arboleda from National University of Colombia for providing scientific and technical support during the experiments. This research was funded by grants from COLCIENCIAS #11565740581 (GPC-G), CODI University of Antioquia 452-2013 (EO), and Scholarship Colciencias call 647 (AMS-G). The content is solely the responsibility of the authors.

Supplementary Figures



Supplementary Figure 1: Linalool reduces the lipid peroxidation in neurons and astrocytes from cortices, but not from hippocampus. (a) Experimental design used in cortical immature neurons, (b) Lipid peroxidation measurement from cortical neurons at DIV 7 exposed to glutamate and different concentrations of Linalool, (c) Experimental design used in hippocampal mature neurons at DIV 19 (d) Dose-response curve of linalool at 0.1 to 200 µM in hippocampal mature neurons, (e) Lipid peroxidation measurement in mature hippocampal neurons, f) Experimental design in astrocytes at DIV 24 (g) Lipid peroxidation in astrocytes from cortex and (h) hippocampus. Lipid peroxidation is expressed as pmol of MDA ((malondialdehyde) per million of neurons or astrocytes.



Supplementary figure 2: Linalool decreases the immunoreactivity of astrocytes and microglia in motor cortex of ischemic rats. (a) GFAP immunoreactivity of the ipsilateral motor cortex area. 10x and 40x magnification. (b) Values in the bar graphs are expressed as relative densitometric units (RU) of GFAP immunoreactivity in the motor cortex (40x) (c) Representative images of Iba-1 immunoreactivity in motor cortex area. (d) Values in the bar graphs are expressed as relative densitometric units (RU) of Iba-1 immunoreactivity (40x). Control: vehicle (PEG); Lin: Linalool. Data are expressed as means ± SEM. n=4-5 per group. ***p<0.001. Scale bar= 50 µm.

Changes in the hippocampal and peripheral phospholipid profiles are associated with neurodegeneration hallmarks in a long-term global cerebral ischemia model: Attenuation by Linalool

References

Adibhatla, R.M., Hatcher, J.F., 2007. Role of lipids in brain injury and diseases. Future Lipidol. 2, 403-422. https://doi.org/10.2217/17460875.2.4.403.

Alinejad, B., Ghorbani, A., Sadeghnia, H.R., 2013. Effects of Combinations of Curcumin, Linalool, Rutin, Safranal, and Thymoquinone on Glucose/Serum Deprivation-induced Cell Death, vol. 3, pp. 321-328.

Api, A.M., Belsito, D., Bhatia, S., Bruze, M., Calow, P., Dagli, M.L., Dekant, W., Fryer, A.D., Kromidas, L., La Cava, S., Lalko, J.F., Lapczynski, A., Liebler, D.C., Miyachi, Y., Politano, V.T., Ritacco, G., Salvito, D., Schultz, T.W., Shen, J., Sipes, I.G., Wall, B., Wilcox, D.K., 2016. RIFM fragrance ingredient safety assessment, α-Ionone. CAS Registry Number 127-41-3 Food Chem. Toxicol. 97, S1-S10. https://doi.org/10.1016/j.fct.2015.12.010.

Barker, M., Rayens, W., 2003. Partial least squares for discrimination. J. Chemom. 17, 166-173. https://doi.org/10.1002/cem.785.

Baskaya, M.K., Prasad, M.R., Donaldson, D., Hu, Y., Rao, A.M., Dempsey, R.J., 1996. Enhanced accumulation of free fatty acids in experimental focal cerebral ischemia. Prostaglandins Leukot. Essent. Fat. Acids 54, 167-171. https://doi.org/10.1016/S0952-3278(96)90012-5.

Batista, P.A., De Paula Werner, M.F., Oliveira, E.C., Burgos, L., Pereira, P., Da Silva Brum, L.F., Story, G.M., Santos, A.R.S., 2010. The antinociceptive effect of (-)-linalool in models of chronic inflammatory and neuropathic hypersensitivity in mice. J. Pain 11, 1222-1229. https://doi.org/10.1016/j.jpain.2010.02.022.

Becerra-Calixto, A., Cardona-Gomez, G.P., 2016. Neuroprotection induced by transplanted CDK5 knockdown astrocytes in global cerebral ischemic rats. Mol. Neurobiol. 1-16. https://doi.org/10.1007/s12035-016-0162-2.

Bermúdez-cardona, J., Velasquez-rodríguez, C., 2016. Phospholipids, cholesterol esters and triglycerides metabolic syndrome. Nutrients 8, 1-13. https://doi.org/10.3390/nu8020054.

Bhuiyan, M.I.H., Kim, H.B., Kim, S.Y., Cho, K.O., 2011. The neuroprotective potential of cyanidin-3-glucoside fraction extracted from mulberry following oxygen glucose deprivation. Korean j. Physiol. Pharmacol. 15, 353-361. https:// doi.org/10.4196/kjpp.2011.15.6.353.

Blusztajn, J.K., Slack, B.E., Mellott, T.J., 2017. Neuroprotective actions of dietary choline. https://doi.org/10.3390/nu9080815.

Bousser, M.G., 2012. Stroke prevention: an update. Front. Med. China 6, 22-34. https://doi.org/10.1007/s11684-012-0178-6.

Celik, S., Ozkaya, A., 2002. Effects of intraperitoneally administered lipoic acid, vitamin E, and linalool on the level of total lipid and fatty acids in Guinea pig brain with oxidative stress induced by H2O2. J. Biochem. Mol. Biol. 35, 547-552.

Cho, S.Y., Jun, H.J., Lee, J.H., Jia, Y., Kim, K.H., Lee, S.J., 2011. Linalool reduces the expression of 3-hydroxy-3-methylglutaryl CoA reductase via sterol regulatory element binding protein-2- and ubiquitin-dependent mechanisms. FEBS Lett. 585, 3289-3296. https://doi.org/10.1016/j.febslet.2011.09.012.

Coelho, V., Mazzardo-Martins, L., Martins, D.F., Santos, A.R.S., Da Silva Brum, L.F., Picada, J.N., Pereira, P., 2013. Neurobehavioral and genotoxic evaluation of (-)-linalool in mice. J. Nat. Med. 67, 876-880. https://doi.org/10.1007/s11418- 013-0751-6.

Davis, C.M., Liu, X., Alkayed, N.J., 2017. Cytochrome P450 eicosanoids in cerebrovascular function and disease. Pharmacol. Ther. 179, 31-46. https://doi.org/10.1016/j.pharmthera.2017.05.004.

Elisabetsky, E., Marschner, J., Onofre Souza, D., 1995. Effects of linalool on glutamatergic system in the rat cerebral cortex. Neurochem. Res. 20, 461-465. https://doi.org/10.1007/BF00973103.

Farooqui, A., 2011. Lipid Mediators and Their Metabolism in the Brain. Springer Science & Business Media.

Farooqui, A.A., 2009. Hot Topics in Neural Membrane Lipidology, Hot Topics in Neural Membrane Lipidology. Springer Science & Business Media, New York. https://doi.org/10.1007/978-0-387-09693-3.

Farooqui, A., Horrocks, L., 2007. Glycerophospholipids in Brain. Phospholipases A2 in Neurological Disorders. Springer.

Garcia, J.H., Wagner, S., Liu, K.F., H, X., 1995. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Stroke 26, 627-634.

George Paxinos, C.W., 2006. The Rat Brain in Stereotaxic Coordinates, sixth ed. Academic Press. 2006.

Gutiérrez-Vargas, J.A., Múnera, A., Cardona-Gomez, G.P., 2015. CDK5 knockdown prevents hippocampal

degeneration and cognitive dysfunction produced by cerebral ischemia. J. Cerebr. Blood Flow Metabol. 35, 1937-1949. https://doi.org/10.1038/jcbfm.2015.150.

Gutierrez Vargas, J., Castro Alvarez, J., Velasquez Carvajal, D., Monta˜nez Velasquez, M., Cespedes Rubio, A., Cardona Gomez, G., 2010. Rac1 activity changes are associated with neuronal pathology and spatial memory long-term recovery after global cerebral ischemia. Neurochem. Int. 57, 762-773. https:// doi.org/10.1016/j. neuint.2010.08.014.

Guzman-Gutierrez, S.L., Bonilla-Jaime, H., Gomez-Cansino, R., Reyes-Chilpa, R., 2015. Linalool and b-pinene exert their antidepressant-like activity through the monoaminergic pathway. Life Sci. 128, 24-29. https:// doi.org/10.1016/j.lfs.2015.02.021.

Jordi Folch, S.S., 1957. A Simple Method for isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509. https://doi.org/10.1016/j. ultrasmedbio.2011.03.005.

Jun, H.-J., Lee, J.-H., Kim, J., Jia, Y., Kim, K.H., Hwang, K.Y., Yun, E.J., Do, K.R., Lee, S.-J., 2014. Aromatic terpenoid linalool is an agonistic ligand for PPARα that reduces plasma triglyceride levels and rewires the hepatic transcriptome and plasma metabolome. J. Lipid Res. 55, 1098-1110. https://doi.org/10.1194/jlr.M045807.

Kim, J., Yin, T., Shinozaki, K., Lampe, J.W., Becker, L.B., 2017. Potential of lysophosphatidylinositol as a prognostic indicator of cardiac arrest using a rat model. Biomarkers 22, 755-763. https://doi.org/10.1080/1354750X.2016.1265002.

Koc, E.R., G€okce, E.C., S€onmez, M.A., Namuslu, M., G€okce, A., Bodur, A.S., 2015. Borax partially prevents neurologic disability and oxidative stress in experimental spinal cord ischemia/reperfusion injury. J. Stroke Cerebrovasc. Dis. 24, 83-90. https://doi.org/10.1016/j. jstrokecerebrovasdis.2014.07.037.

Koizumi, S., Yamamoto, S., Hayasaka, T., Konishi, Y., Yamaguchi-Okada, M., Goto- Inoue, N., Sugiura, Y., Setou, M., Namba, H., 2010. Imaging mass spectrometry revealed the production of lyso-phosphatidylcholine in the injured ischemic rat brain. Neuroscience 168, 219-225. https://doi.org/10.1016/j.neuroscience.2010.03.056.

Li, J., Zhang, X., Huang, H., 2014. Protective effect of linalool against lipopolysaccharide/d-galactosamine-induced liver injury in mice. Int. Immunopharm. 23, 523-529. https://doi.org/10.1016/j.intimp.2014.10.001.

Li, X.J., Yang, Y.J., Li, Y.S., Zhang, W.K., Bin, Tang, H., 2016. ??-Pinene, linalool, and 1- octanol contribute to the topical anti-inflammatory and analgesic activities of frankincense by inhibiting COX-2. J. Ethnopharmacol. 179, 22-26. https://doi.org/10.1016/j.jep.2015.12.039.

Li, Y., Lv, O., Zhou, F., Li, Q., Wu, Z., Zheng, Y., 2015. Linalool inhibits LPS-induced inflammation in BV2 microglia cells by activating Nrf2. Neurochem. Res. 40, 1520-1525. https://doi.org/10.1007/s11064-015-1629-7.

Liu, X., Moon, S.H., Jenkins, C.M., Sims, H.F., Gross, R.W., 2016. Cyclooxygenase-2 mediated oxidation of 2-arachidonoyl-lysophospholipids identifies unknown lipid signaling pathways. Cell Chem. Biol. 23, 1217-1227. https://doi.org/10.1016/j.chembiol.2016.08.009.

Martinez-Gardeazabal, J., Gonzalez de San Roman, E., Moreno-Rodríguez, M., LLorente-Ovejero, A., Manuel, I., Rodríguez-Puertas, R., 2017. Lipid mapping of the rat brain for models of disease. Biochim. Biophys. Acta 1859, 1548-1557. https://doi.org/10.1016/j.bbamem.2017.02.011.

Mateos, M.V., Salvador, G.A., Giusto, N.M., 2010. Selective localization of phosphatidylcholine-derived signaling in detergent-resistant membranes from synaptic endings. Biochim. Biophys. Acta Biomembr. 1798, 624-636. https://doi. org/10.1016/j.bbamem.2009.12.008.

McBean, D.E., Kelly, P.A.T., 1998. Rodent models of global cerebral ischemia: a comparison of two-vessel occlusion and four-vessel occlusion. Gen. Pharmacol. 30, 431-434. https://doi.org/10.1016/S0306-3623(97)00284-X.

Mehri, S., Meshki, M.A., Hosseinzadeh, H., 2015. Linalool as a neuroprotective agent against acrylamide-induced neurotoxicity in Wistar rats. Drug Chem. Toxicol. 38, 162-166. https://doi.org/10.3109/01480545.2014.919585.

Miyashita, M., Sadzuka, Y., 2013. Effect of linalool as a component of Humulus lupulus on doxorubicin-induced antitumor activity. Food Chem. Toxicol. 53, 174-179. https://doi.org/10.1016/j.fct.2012.11.035.

Miyawaki, S., Imai, H., Hayasaka, T., Masaki, N., Ono, H., Ochi, T., Ito, A., Nakatomi, H., Setou, M., Saito, N., 2016. Imaging mass spectrometry detects dynamic changes of phosphatidylcholine in rat hippocampal CA1 after transient global ischemia. Neuroscience 322, 66-77. https://doi.org/10.1016/j.neuroscience.2016.02.013.

Monteiro, J.P., Oliveira, P.J., Jurado, A.S., 2013. Mitochondrial membrane lipid remodeling in pathophysiology: a new target for diet and therapeutic interventions. Prog. Lipid Res. 52, 513-528. https://doi.org/10.1016/j. plipres.2013. 06.002.

Moskowitz, M.A., Lo, E.H., Iadecola, C., 2010. The science of stroke: mechanisms in search of treatments. Neuron 67, 181-198. https://doi.org/10.1016/j.neuron. 2010.07.002.

Niki, E., 2014. Biomarkers of lipid peroxidation in clinical material. Biochim. Biophys. Acta Gen. Subj. 1840, 809-817. https://doi.org/10.1016/j.bbagen.2013.03. 020.

Park, H., Seol, G.H., Ryu, S., Choi, I.Y., 2016. Neuroprotective effects of (-)-linalool against oxygen-glucose deprivation-induced neuronal injury. Arch Pharm. Res. (Seoul) 39, 555-564. https://doi.org/10.1007/s12272-016-0714-z.

Park, S.N., Lim, Y.K., Freire, M.O., Cho, E., Jin, D., Kook, J.K., 2012. Antimicrobial effect of linalool and α-terpineol against periodontopathic and cariogenic bacteria. Anaerobe 18, 369-372. https://doi.org/10.1016/j.anaerobe.2012.04.001.

Parpura, V., Fisher, E.S., Lechleiter, J.D., Schousboe, A., Waagepetersen, H.S., Brunet, S., Baltan, S., Verkhratsky, A., 2016. Glutamate and ATP at the interface between signaling and metabolism in Astroglia: examples from pathology. Neurochem. Res. https://doi.org/10.1007/ s11064-016-1848-6.

Peana, A.T., Marzocco, S., Popolo, A., Pinto, A., 2006. (-)-Linalool inhibits in vitro NO formation: probable involvement in the antinociceptive activity of this mono-terpene compound. Life Sci. 78, 719-723. https:// doi.org/10.1016/j.lfs.2005.05. 065.

Phillis, J.W., O'Regan, M.H., 2004. A potentially critical role of phospholipases in central nervous system ischemic, traumatic, and neurodegenerative disorders. Brain Res. Rev. 44, 13-47. https://doi.org/10.1016/j. brainresrev.2003.10.002.

Pilitsis, J.G., Diaz, F.G., O'Regan, M.H., Phillis, J.W., 2002. Differential effects of phospholipase inhibitors on free fatty acid efflux in rat cerebral cortex during ischemia-reperfusion injury. Brain Res. 951, 96-106. https://doi.org/10.1016/ S0006-8993(02)03142-6.

Posada-Duque, R.A., Palacio-Castañeda, V., Cardona-Gómez, G.P., 2015. CD5 knockdown in astrocytes provide neuroprotection as a trophic source via Rac1. Mol. Cell. Neurosci. 68, 151-166. https://doi.org/10.1016/j.mcn.2015.07.001.

Posada-Duque, R.A., Velasquez-Carvajal, D., Eckert, G.P., Cardona-Gomez, G.P., 2013. Atorvastatin requires geranylgeranyl transferase-I and Rac1 activation to exert neuronal protection and induce plasticity. Neurochem. Int. 62, 433-445. https://doi.org/10.1016/j. neuint.2013.01.026.

Rabiei, Z., Reza, M., Rasoulian, B., Ghassempour, A., 2012. Phytomedicine the neuroprotection effect of pretreatment with olive leaf extract on brain lipidomics in rat stroke model. Eur. J. Integr. Med 19, 940-946. https://doi.org/10.1016/j. phymed.2012.06.003.

Raguso, R.A., 2016. More lessons from linalool: insights gained from a ubiquitous floral volatile. Curr. Opin. Plant Biol. 32, 31-36. https://doi.org/10.1016/j.pbi. 2016.05.007.

Richter, M., Nickel, C., Apel, L., Kaas, A., Dodel, R., Culmsee, C., Dolga, A.M., 2015. Neurochemistry International SK channel activation modulates mitochondrial respiration and attenuates neuronal HT-22 cell damage induced by H2O2. Neurochem. Int. 81, 63-75. https:// doi.org/10.1016/j.neuint.2014.12.007.

Rodenak Kladniew, B., Polo, M., Montero Villegas, S., Galle, M., Crespo, R., García De Bravo, M., 2014. Synergistic antiproliferative and anticholesterogenic effects of linalool, 1,8-cineole, and simvastatin on human cell lines. Chem. Biol. Interact. 214, 57-68. https://doi. org/10.1016/j.cbi.2014.02.013.

Rothwell, P.M., Algra, A., Amarenco, P., 2011. Medical treatment in acute and long-term secondary prevention after transient ischaemic attack and ischaemic stroke. Lancet 377, 1681-1692. https://doi.org/10.1016/ S0140-6736(11)60516-3.

Sabogal-Guáqueta, A.M., Osorio, E., Cardona-Gómez, G.P., 2016. Linalool reverses neuropathological and behavioral impairments in old triple transgenic Alzheimer's mice. Neuropharmacology 102, 111-120. https:// doi.org/10.1016/j. neuropharm.2015.11.002.

Sasaki, T., Sasaki, J., Sakai, T., Takasuga, S., Suzuki, A., 2007. The physiology of phosphoinositides. Biol. Pharm. Bull. 30, 1599-1604. https://doi.org/. https:// doi.org/10.1248/bpb.30.1599.

Schulz, U.G., 2013. Drug treatments in the secondary prevention of ischaemic stroke. Maturitas 76, 267-271. https://doi.org/10.1016/j.maturitas.2013.05.009.

Shi, F., Zhao, Y., Firempong, C.K., Xu, X., 2016. Preparation, characterization and pharmacokinetic studies of linalool-loaded nanostructured lipid carriers. Pharm. Biol. 54, 2320-2328. https://doi.org/10.3109/13880209.2016.1155630.

Singh, H., Usher, S., Poulos, A., 1989. Mitochondrial and peroxisomal beta-oxidation of stearic and lignoceric acids by rat brain. J. Neurochem. 53, 1711-1718. https://doi.org/10.1111/j.1471-4159.1989.tb09235.x.

Singh, R.P.A.L., Singh, I., 1986. Peroxisomal Oxidation of Lignoceric Acid in Rat Brain, vol. 11, pp. 281-289.

Tian, H., Qiu, T., Zhao, J., Li, L., Guo, J., 2009. Sphingomyelinase-induced ceramide production stimulate calcium-independent JNK and PP2A activation following cerebral ischemia. Brain Inj. 23, 1073-1080. https://doi.org/10.3109/ 02699050903379388.

Tomimoto, H., Akiguchi, I., Wakita, H., Lin, J.X., Budka, H., 2000. Cyclooxygenase-2 is induced in microglia during chronic cerebral ischemia in humans. Acta Neuropathol. 99, 26-30. https://doi.org/10.1007/ PL00007402.

Toschi, V., Motta, a, Castelli, C., Paracchini, M.L., Zerbi, D., Gibelli, a, 1998. High prevalence of antiphosphatidylinositol antibodies in young patients with cerebral ischemia of undetermined cause. Stroke 29, 1759-1764. https://doi.org/ 10.1161/01.STR.29.9.1759.

Vicinanza, M., D 'angelo, G., Campli, A. Di, De Matteis, M.A., 2008. Function and dysfunction of the PI system in membrane trafficking. EMBO J. 27, 2457-2470. https://doi.org/10.1038/emboj.2008.169.

Villamil-Ortiz, J.G., Barrera-Ocampo, A., Piedrahita, D., Velásquez-Rodríguez, C.M., Arias-Londoño, J.D., Cardona-Gómez, G.P., 2016. BACE1 RNAi restores the composition of phosphatidylethanolamine-derivates related to memory improvement in aged 3xTg-AD mice. Front. Cell. Neurosci. 10, 1-14. https://doi.org/10.3389/ fncel.2016.00260

Villamil Ortiz, J.G., Cardona Gomez, G.P., 2015. Comparative analysis of autophagy and tauopathy related markers in cerebral ischemia and Alzheimer's disease animal models. Front. Aging Neurosci. 7, 1-14. https://doi.org/10.3389/fnagi. 2015.00084.

Wang, C.X., Shuaib, A., 2002. Involvement of inflammatory cytokines in central nervous system injury. Prog. Neurobiol. 67, 161-172. https://doi.org/10.1016/S0301-0082(02)00010-2.

Whiley, L., Sen, A., Heaton, J., Proitsi, P., García-Gómez, D., Leung, R., Smith, N., Thambisetty, M., Kloszewska, I., Mecocci, P., Soininen, H., Tsolaki, M., Vellas, B., Lovestone, S., Legido-Quigley, C., 2014. Evidence of altered phosphatidylcholine metabolism in Alzheimer's disease. Neurobiol. Aging 35, 271-278. https://doi.org/10.1016/j.neurobiolaging.2013.08.001.

Xian, W., Wu, Y., Xiong, W., Li, L., Li, T., Pan, S., Song, L., Hu, L., Pei, L., Yao, S., Shang, Y., 2016. The pro-resolving lipid mediator Maresin 1 protects against cerebral ischemia/reperfusion injury by attenuating the pro-inflammatory response. Biochem. Biophys. Res. Commun. 472, 175-181. https://doi.org/10. 1016/j.bbrc.2016.02.090.

Xu, P., Wang, K., Lu, C., Dong, L., Gao, L., Yan, M., Aibai, S., Yang, Y., Liu, X., 2017. Protective effects of linalool against amyloid beta-induced cognitive deficits and damages in mice. Life Sci. 174, 21-27. https://doi.org/10.1016/j.lfs.2017.02.010.

Yamaji-Hasegawa, A., Tsujimoto, M., 2006. Lipid dynamics and pathobiology in membrane lipid rafts asymmetric distribution of phospholipids in bio-membranes. Biol. Pharm. Bull. 29, 1547-1553. https://doi.org/10.1248/bpb.29. 1547.

Yi, X., Han, Z., Zhou, Q., Lin, J., Liu, P., 2016. 20-Hydroxyeicosatetraenoic acid as a predictor of neurological deterioration in acute minor ischemic stroke. Stroke 47, 3045-3047. https://doi.org/10.1161/STROKEAHA.116.015146.

Changes in the hippocampal and peripheral phospholipid profiles are associated with neurodegeneration hallmarks in a long-term global cerebral ischemia model: Attenuation by Linalool

CHAPTER 4

Linalool attenuates oxidative stress and mitochondrial dysfunction mediated by glutamate and NMDA toxicity

Angélica María Sabogal-Guáqueta[a,b,c], , Fabian Hobbie[a] , Akshaya Keerthi[a] , Asmaa Oun[a,d] , Arjan Kortholt[d] , Erik Boddeke[c] , Amalia Dolga[a]

[a] Department of Molecular Pharmacology, Faculty of Science and Engineering, Groningen Research Institute of Pharmacy, Behavioral and Cognitive Neurosciences (BCN), University of Groningen, Groningen, the Netherlands

[b] Biomedical Sciences of Cells & Systems, Molecular Neurobiology, Faculty of Medical Sciences, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

[c] Neuroscience Group of Antioquia, Cellular and Molecular Neurobiology Area- School of Medicine, SIU, University of Antioquia, Medellín, Colombia

[d] Department of Cell Biochemistry, Groningen Institute of Biomolecular Sciences & Biotechnology, University of Groningen, Groningen, the Netherlands

Biomedicine & Pharmacotherapy 118 (2019) 1-12. doi: 10.1016/j.biopha.2019.109295

Abstract

Mitochondrial dysfunction and inflammation contribute to the initiation and development of several brain pathological conditions, including Alzheimer's disease and cerebral ischemia. Linalool is an aromatic plant-derived monoterpene alcohol with reported anti-inflammatory, and anti-oxidant properties. We investigated the role of linalool on glutamate-induced mitochondrial oxidative stress in immortalized neuronal HT-22 cells. Glutamate induced oxidative stress in neuronal cells, as detected by real-time cell impedance measurements, MTT assay, and analysis of Annexin V/PI. Administration of linalool 100 µM reduced cell death mediated by glutamate. Staining of glutamate-stimulated mitochondria by MitoTracker revealed improved morphology in the presence of linalool. Furthermore, we demonstrated a potential neuroprotective effect of linalool in conditions of oxidative stress by a reduction of mitochondrial ROS and mitochondrial calcium levels, and by preserving mitochondrial membrane potential. Experiments using both high-resolution respirometry and Seahorse Extracellular flux analyzer showed that linalool was able to promote an increase in uncoupled respiration that could contribute to its neuroprotective capacity. Linalool protection was validated using organotypic hippocampal slices as *ex vivo* model with NMDA as a stimulus to induce excitotoxity cell damage. These results demonstrate that linalool is protective in an *in vitro* model of glutamate-induced oxidative stress and in an *ex-vivo* model for excitotoxity, proposing linalool as a potential therapeutic agent against neurodegenerative brain diseases where oxidative stress contributes to the pathology of the disease.

Keywords: Oxidative stress, linalool, OHSC, mitochondria, neuroprotection

Linalool attenuates oxidative stress and mitochondrial dysfunction mediated by glutamate damage

1. Introduction

Oxytosis is a non-apoptotic form of cell death associated with increased oxidative stress and, according to recent findings, also with mitochondrial dysfunction [1,2]. The term oxytosis was first introduced on the basis of findings on oxidative cell death in neuronal cells [3]. Oxytosis is characterized by both cellular and mitochondrial ROS accumulation [4]. Emerging evidence links oxytosis cell death to the field of neuroscience with increasing indication for its contribution to brain injury and degeneration [5]. For example, oxytosis cell death mechanisms contribute to the pathology of many age-associated neuro-degenerative diseases such as Alzheimer disease (AD) or acute events such as cerebral ischemia, where elevated production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), causes damage to all components of the cell, including proteins, lipids, and DNA [6–8].

Oxytosis-induced cell death can be initiated by compounds inhibiting the cysteine/glutamate antiporter system (x_c^-), such as glutamate [3,9–11]. Blockage of the x_c^- system by excessive extracellular glutamate prevents the import of cystine, thereby reducing substrate availability for glutamate cysteine ligase (GCL), leading to depletion of intracellular glutathione (GSH) and decreased activity of glutathione peroxidase 4 (GPX4) [5]. GSH plays an essential role in several physiological processes acting directly as a co-factor in enzymatic reactions or indirectly as the major thiol-disulfide redox buffer in all mammalian cells. These properties allow GSH to provide a cellular critical defense system against many forms of cellular stress, in particular, oxidative stress [12]. Reduced levels of cystine, in response to excessive glutamate application, leads to a depletion of GSH, GPX4 inhibition, and lipid peroxide formation. These events result in an increase of mitochondrial and cellular ROS levels, resulting in cell death.

Glutamate-mediated oxidative stress mechanisms have been extensively studied in HT-22 cells, a subclone of the hippocampal cell line HT4 [13]. This neuronal cell line has been specifically selected for its sensitivity to glutamate. Exposure of neuronal HT-22 cells to toxic concentrations of glutamate initiates a well-defined signaling cascade which mediates cell death through inhibition of xc− GSH depletion, excessive ROS production and mitochondrial damage [5]. Recent evidence supports that natural compounds (monoterpenes) might prevent oxidative stress, pathological features of neurodegenerative diseases and of acute events such as stroke [14,15]. Linalool, an enantiomer of the naturally occurring, aromatic plant-derived (e.g. *Lavandula angustifolia, Melissa officinalis, Rosmarinus officinalis,* and *Cymbopogon citratus*) monoterpene alcohol is a major component of essential oils [16]. Linalool exhibits several effects on the CNS, serving as antinociceptive, anticonvulsant, anxiolytic, and sedative agent [17–19]. Furthermore, various pharmacological properties such as antimicrobial, anti-inflammatory, antileishmanial, anti-atherogenic, and anti-depressant effects have been reported [20–25]. Antioxidant properties of linalool were also demonstrated

in a study, investigating oxidation reactions in unsaturated fatty acids extracted from guinea pig brains challenged with H_2O_2 to induce oxidative stress [26].

We have recently shown linalool being protective in neurodegenerative diseases, and being able to reduce the histopathological markers, including amyloid plaques and tau hyperphosphorylation in a triple transgenic mice model of AD (3xTg-AD) with improvements in behavioral assessments of learning and spatial memory [27]. Besides, in an *in vitro* model of glutamate excitotoxicity, we found that linalool protected neurons and astrocytes as indicated by reduced lactate de-hydrogenase (LDH) release as well as recovered cellular ATP levels, a marker of improved mitochondrial functions. Moreover, neurological, motor, and cognitive impairments were ameliorated by oral linalool administration following global ischemia [28].

Linalool has been shown protective in several models of neurodegeneration, however, the mechanisms underlying the neuroprotective effect of linalool in relation to mitochondrial functions still remain elusive and additional research is necessary in order to determine its precise effects.

Our goal was to evaluate the effect of linalool on the physiological function of mitochondria and its potential neuroprotective properties in conditions of glutamate-induced oxytosis in HT-22 cells and in *ex vivo* organotypic hippocampal slices.

2. Materials and methods

2.1. Cell culture

HT-22 cells (kindly provided by Prof. Culmsee), which were used to model neurons, were cultured in Dulbecco's modified Eagle Medium (DMEM; Life Technologies, UK, #42340-025) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Utah, USA, #SV30160.03), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco; Life Technologies, USA, #15070-063) and 1% sodium pyruvate (Gibco, Life Technologies Corporation, USA, #11360-070) at 37°C and 5% CO_2. The passage number used were between 200-250 and the cells were mycoplasma negative. Additional substances used were linalool (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #L2602, Lot# STBG7505), and glutamate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #G1626, Lot#slbl6389 V).

2.2. Cell viability assay and xCELLigence measurement

Cell viability and metabolic activity of cells were investigated using the colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reduction assay. MTT solution (0.5 mg/ml) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany #M5655) was administered to cells in a 96-well plate format for maximum 1 h. Afterward, the MTT-containing medium was entirely removed and the plate was incubated for at least 2–4 h at −80 °C. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #D8418, Lot# SHBH9942) was applied to the cells which allowed the formazan to uniformly disperse in the solution. DMSO was incubated for 1 h at 37°C under continuous shaking conditions. The absorbance values were determined by Synergy™H1 (Bad Friedrichshall, Germany) Hybrid Multi-Mode Reader at 570 nm with a reference filter at 630 nm. Untreated healthy cells, defined as controls, were regarded as 100% cell viability. We dissolved the linalool in DMSO 0.1%, and this solvent had no effect on cellular activity or metabolic activity. HT-22 cells were co-treated with linalool or glutamate seeded onto 96 well plates in conditions as follows: control condition, linalool 100 µM, glutamate 6 mM and co-treatment with glutamate 6 mM plus linalool 100 µM.

Cell viability was measured in real-time using the xCELLigence ®RTCA MP system (ACEA BIO, The Netherlands) which is a label-free cell-based assay suitable for continuous monitoring of biological processes of living cells [29]. Continuous cell monitoring was performed using an E-plate 96 and measured by the xCELLigence ®RTCA MP Instrument. Cellular impedance was assessed every 30 min for 24 h and represented as Normalized Cell Index (NCI) which was defined before the application of the experimental conditions (control, linalool 100 µM, glutamate 6 mM and co-treatment of glutamate 6 mM with linalool 100 µM) as the starting point (t: 0 h) of the experiment.

2.3.Flow cytometry

Several intracellular parameters associated with oxidative stress, mitochondrial dysfunction, and cell death were determined by flow cytometry analysis using the CytoFLEXS benchtop flow cytometer (Beckman Coulter Life Sciences, Indianapolis, USA). HT-22 cells were seeded in 24-well plates with 40,000 cells/well. To allow the comparison between different treatment groups (control condition, linalool 100 µM, glutamate 6 mM, and co-treatment of glutamate 6 mM with linalool 100 µM) and the total amount of cells per condition. The supernatant of the medium was collected and washed with PBS, followed by trypsinization. For every condition, three wells were analyzed and the same number of cells (10,000)/well was counted by flow cytometry. Minimum of 3 independent experiments were performed, and the quantification of data was acquired using Kaluza Analysis 1.5 software.

2.3.1.Annexin V/PI

In order to assess the amount of apoptotic as well as necrotic cells after stimulation, Annexin V FITC and propidium iodide (PI) (Invitrogen, Oregon, USA, #V13242, Lot#2008168) double staining kit was used. After treatment, HT-22 cells were trypsinized, and the cell suspension was incubated with Annexin V FITC and PI in binding buffer for 10 min at room temperature (RT). Detection of fluorescence was acquired at FITC: 494/518 and PI: 535/617.

Linalool attenuates oxidative stress and mitochondrial dysfunction mediated by glutamate damage

2.3.2. Mitochondrial ROS production

Mitochondrial ROS formation was evaluated by the MitoSOX dye (Invitrogen, Oregon, USA, #M36008, Lot#1924466). HT-22 cells were incubated with MitoSOX™ 1.25 µM dye for 30 min at 37 °C. Fluorescence was excited at 488 nm and detected at 690/50 nm.

2.3.3.Mitochondrial membrane potential

Loss of mitochondrial membrane potential (ΔΨm) was determined by staining with TMRE™ dye (tetramethylrhodamine-ethyl ester; Invitrogen, Oregon, USA, #T669). Cells were collected and incubated for 20 min with TMRE 0.2 µM at 37 °C. Fluorescence was excited at 488 nm and detected at 690/50 nm.

2.3.4.Measurement of mitochondrial calcium

For the measurement of mitochondrial calcium levels, HT-22 cells were incubated with of rhodamine-2-acetoxymethylester 2 µM dye (Rho2-AM) (Abcam, #ab142780, Lot# APN15176-1-1) in DMEM without serum for 30 min at RT, followed by incubation in DMEM for 30 min at RT in the dark and washed with PBS. Cells were excited at 552 nm wavelength and the emission intensities at 581 nm.

2.3.4.Lipid peroxidation

Lipid peroxidation was estimated with BODIPY 2 µM staining dye (Invitrogen, Karlsruhe, Germany, #D3861, Lot# 1890330) for 60 min at 37 °C. Cells were washed once with PBS followed by their analysis using the excitation channel at 488 nm and detection channel at 530 nm.

2.4. Mitochondrial morphology: Mito Tracker deep red

HT-22 cells (4×10^4 cells) were treated with different conditions (control condition, linalool 100 µM, glutamate 6 mM and co-treatment of glutamate 6 mM with linalool 100 µM) for 18 h and incubated with 200 nM MitoTracker™Deep Red FM (Invitrogen, Oregon, USA, #M22426, Lot# 509441) for 30 min at 37°C. After fixation with 4% Paraformaldehyde (PFA), the fluorescence was excited at 620 ± nm and detected at 700 ± 75 nm. 500 cells per condition were counted and classified into four different categories based on their mitochondrial morphology. This categorization was obtained by an investigator with no prior knowledge of the experimental conditions.

Category I represents healthy cells where mitochondria form an elongated tubular network distributed equally throughout the cytosol, Category II represents semi-viable cells comprising short yet tubular mitochondrial networks around the cytosol. Category II comprises mitochondria that appear short but are still forming long tubules. Cells which entail large, fragmented/ granulated mitochondria around the nucleus are referred as Category III. These cells do not show apoptotic features. On the contrary, damaged and dying cells were identified by small rounded mitochondria of different sizes within proximity

of the nucleus. The latter mitochondria are referred to as Category IV [30]. Three independent experiments were performed.

2.5. Mitochondrial respiration

HT-22 cells ($4–8 \times 10^7$) were harvested and mitochondria were isolated utilizing semi-automated pump-controlled cell rupture system (0.71 ml/min) (PCC) using syringes (SGE, Trajan© Scientific, Australia) attached to a cell homogenizer (Isobiotech, EMBL Heidelberg, Germany) superimposed on a pump (ProSense, Oosterhout, NL, #NE-1000) [31]. Mitochondria were suspended in isolation buffer (sucrose 250 mM, HEPES 20 mM, EDTA 3 mM adjusted to pH 7,5). The entire procedure was performed on ice.

2.5.1. High-resolution respirometry (Oroboros)

The amount of protein derived from the isolation procedure was 250 µg and it was determined using the Pierce™BCA Protein Assay Kit (Pierce™BCA Protein Assay Kit, Thermo Scientific, Rockford, USA, #23225). Mitochondrial respiration, of the organelles obtained from the HT-22, was analyzed via high-resolution respirometry oxygraph O2K (Oroboros Systems, Innsbruck, Austria). The isolated mitochondria prepared as previously described [31] were treated with linalool 100 µM or ethanol for 25 min, followed by Oroboros measurements. Mitochondrial state 1 was monitored under continuous stirring at 750 rpm in 1 ml MiR05 buffer (EGTA 0.5 mM, $MgCl_2$ 3 mM, lactobionic acid 60 mM, taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, D-Sucrose 110 mM, BSA, essential fatty acid-free1g/L, at 7.4 pH). In order to perform the oxygen polarography, DatLab software (Oroboros Systems, Innsbruck, Austria) was implemented to record real-time oxygen flux per tissue mass (pmol $O2.s^{-1} * mg^{-1}$) at 37 °C. Non-phosphorylating respiration (State 2) was assessed by addition of complex-I linked substrates (5 mM) pyruvate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany #P-2256), and (2 mM) malate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #374318). Thereafter, the oxidative-phosphorylation capacity (OXPHOS, State 3) of complex-I linked activity or basal respiration was initiated by means of adding saturating concentration (0.8 mM) of ADP (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #A5285). Subsequently, addition of oligomycin 0.1 µM/ml (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #O4876) was used to block *Fo* of the ATP-synthase, thereby inhibiting proton passage. Besides, maximal respiration (State 3 u) was assessed by incremental (steps 1 µM) addition of the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #C2920). End of measurement by addition of Sodium dithionite (DTT, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #15,795-3) to react with all the oxygen present in the chamber. The oxygen concentration as well as the initial derivate of the oxygen concentration which is a derivative of the oxygen consumption is provided as the O 2 slope (pmol*(mL/s) of raw isolated mitochondria. The measurements were recorded in intervals (2 s) using instrumental background correction after calibration of the polarographic oxygen sensors.

2.5.2. Measurement of cellular oxygen consumption rate (OCR)

Oxygen consumption rate (OCR) was measured using XFe96 extra-cellular flux analyzer (Seahorse Bioscience, Billerica, MA) with the mitochondrial stress test as previously described [1]. HT-22 cells were seeded in XFe96-well cell culture microplate at a density of 10,000 cell/ well and incubated at 37 °C incubator with 5% CO_2. 24 h before the assay, the growth medium was replaced with a new medium containing linalool 100 µM or 200 µM. Cells were incubated for 1 h in 180 µL of assay medium (non-buffered DMEM) supplemented with 4.5 g/L glucose, 2 mM glutamine and 1 mM pyruvate, pH 7.35 in CO 2 free incubator at 37 °C. During the assay, basal respiration was measured before injecting the following inhibitors: oligomycin (4 µM; port A) as an ATP synthase inhibitor; 2,4-Dinitrophenol (DNP) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #34334) (50 µM; port B) as a mitochondrial oxidative phosphorylation uncoupler; and rotenone (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #R8875) /antimycin A (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #A8674) 0.1 µM and 1 µM; port C) as complex I and complex III inhibitors. The OCR measurements were normalized to the protein amount in each well using BCA assay. Three independent experiments with 6 wells/condition were performed, and One-way ANOVA test was used to determine statistical significance between different groups.

2.6. Animals

C57BL/6 J mice from the central animal laboratory at University of Groningen were housed and handled in accordance to Dutch standards and guidelines. All experiments were approved by the University of Groningen Committee for Animal Experimentation.

2.6.1. Organotypic hippocampal slice cultures and Maintenance

Organotypic hippocampal slice cultures (OHSC) were prepared from P0-3 mouse pups according to the mentioned protocol with slight modifications [32]. Briefly, after quick decapitation, brains were removed and the hippocampi were dissected out in ice cold serum-free Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #14170-088, Lot#1997485), supplemented with 0.5% glucose (Sigma) and 15 mM HEPES. Isolated hippocampi were transversally cut into 350–375 µm thick slices using a McIlwain tissue chopper. OHSC were then transferred onto Millcell permeable membranes (0.4 µm pore size, Millipore, PICM03050) in six well plates. These culture plate inserts, containing 5 to 6 slices with culture medium: 0.5% Minimum essential medium- MEM (Gibco, #21430-020,), 25% Basal Medium Eagle (BME) (Gibco, 41010-026), 25% heat-inactivated horse serum (Gibco, 16050-122) supplemented with 0.65% glucose (Sigma, G8769) and 2 mM glutamax (Gibco, 35050-038), pH: 7.2. OHSC were maintained at 35°C, 5% CO_2 and with culture medium being changed the first day after preparation and every second day. Experiments were performed after 7–8 days *in vitro*.

2.6.2. Drug treatment and cell damage in OHSCs

To induce excitotoxicity, OHSCs were exposed to 10 µM N-Methyl-D-aspartic acid (NMDA) ((Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #M3262) for 24 h (n:5). At the same time, the OHSCs were exposed to linalool 100 µM and a combination of NMDA with linalool 100 µM with its respective control. Following 8 days *in vitro* (DIV8), slice viability was determined using propidium iodide (PI) staining (5 µg/ml; Sigma- Aldrich, 81845). PI uptake was recorded using a Confocal Leica sp8 fluorescence microscope and analyzed by densitometry using Fiji-ImageJ software. The 3 main areas of the hippocampus where the analysis was performed included CA 1 (Cornu Ammonis 1), CA3 (Cornu Ammonis 3) and DG (Dentate gyrus).

2.6.3. RNA Extraction and quantitative real-time reverse- transcription polymerase chain reaction (RT-qPCR)

For RNA extraction, 6 OHSC per condition were pooled (one sample) and incubated in 500 µl using TriReagent (Invitrogen, Life Technologies, #AM9673) and trimethylene chlorobromide (BCP) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #B9673) followed by washing in isopropanol and elution in 30 µl RNAse-free ddH_2O (n:4-6). The concentration and purity of the RNA was determined by measuring absorbance at 230, 160, and 280 nm with a Nanodrop ND-1000 spectrophotometer. Samples were considered to be sufficiently free of protein and phenol contamination and suitable for PCR analysis when the 260/280 and 260/230 ratios were between 1.8 and 2.2.

Total RNA was transcribed into cDNA using reverse transcription by AMV Reverse Transcriptase Kit (Promega, Madison, WI, #A3500) and was diluted with ddH2O proportionately according to concentration of input RNA. RT-qPCR was performed using an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories GmbH) in the presence of SYBR Green (Roche, #04913914001) using Illumina Eco Personal qPCR System (Westburg, Leusden, The Netherlands). PCR cycling was follows: 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, for 45 cycles. qRT-PCR data were analyzed with LinRegPCR analysis software [33]. Geometric mean of the reference genes ribosomal protein L13A (Rpl3A) and ribosomal RNA 18S was used to normalize qRT-PCR data. Mouse mRNA primers used in analysis were purchased from Biolegio and the sequences are listed in Table 1.

2.7. Statistical analysis

Statistical significance was evaluated using the unpaired Student´s t-test or ANOVA and Tukey`s test for post hoc multiple comparisons of the parametric data in between-group analyses. Non-parametric data were evaluated using Kruskal- Wallis test. Data were analyzed using GraphPad Prism software (version 7.0, GraphPad Software Inc., La Jolla, CA, USA),

Table 1. List of primers used for qPCR experiments

Gene	Forward	Reverse
Bax	AACTGGTGCTCAAGGCCC	TCCCGAAGTAGGAGAGGAG
Cox-2	TGAGTACCGCAAACGCTTC	CAGCCATTTCCTTCTCTCC
Il-1β	TGCCACCTTTTGACAGTGA	ATGTGCTGCTGCGAGATTT
Nos2	GGGACTGAGCTGTTAGAGACAC	TCTTGTATTGTTGGGCTGAGAACA
Rpl3A	AGAAGCAGATCTTGAGGTTACGG	GTTCACACCAGGAGTCCGTT
18S	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA
Tnf-α	TACTGAACTTCGGGGTGATTGGTCC	CAGCCTTGTCCCTTGAAGAGAACC

expressed as mean ± SD for xCELLigence measurements and SEM for the rest experiments. *P* values indicating statistical significance differences between mean values are defined as follows: *p < .05, **p< .01, ***p < .001.

3. Results

3.1. Linalool ameliorated cytotoxicity and apoptosis in glutamate-stimulated HT-22 cells

Linalool had been reported protective in *in vitro* models of gluta-mate-induced excitotoxicity in primary cortical neurons [28]. Here, we evaluated the effect of linalool on glutamate-induced oxidative stress in neuronal HT-22 cells [2]. At first, the potential protection against glutamate-induced oxytosis was assessed using various linalool concentrations. Based on the previous experiments, we choose 100 µM concentration of linalool to investigate the underlying molecular neuroprotective mechanisms of linalool. Following the initiation of oxytosis, HT-22 cells change their morphological shape from the healthy spindle-shaped morphology to a more round-shaped morphology, culminating with the detachment of the cells from the bottom of the cell culture dish (Fig. 1a, upper panels). The protective effect of linalool was demonstrated by cell viability measurements using MTT assay following 18 h of glutamate challenge in the presence of linalool. We observed that glutamate stimulation leads to a significant cell death, whereas co-treatment with linalool in addition to glutamate significantly reduces cell death when compared to the glutamate condition. Application of linalool alone to cells did not significantly affect HT-22 cell viability at concentrations of 200 to 400 µM, however, 500 µM reduced cell viability (Fig. 1.b). Moreover, we observed that linalool at low nanomolar ranges did not mediate neuroprotection (Figure suppl 1.a). In addition, we performed pre-treatment experiments with 2 h or 8 h of linalool and detected that both pretreatment with linalool for 8 h and 2 h followed by glutamate challenge provided protection against glutamate toxicity. Interestingly, the presence of linalool during the glutamate challenge was a prerequisite for the observed protection, since the removal of linalool after the pretreatment was not able to mediate protection against glutamate (Figure suppl 1.b). To further confirm these neuroprotective effects of linalool, we quantified cell death by Annexin V/PI propidium iodide staining. Cells were treated with glutamate,

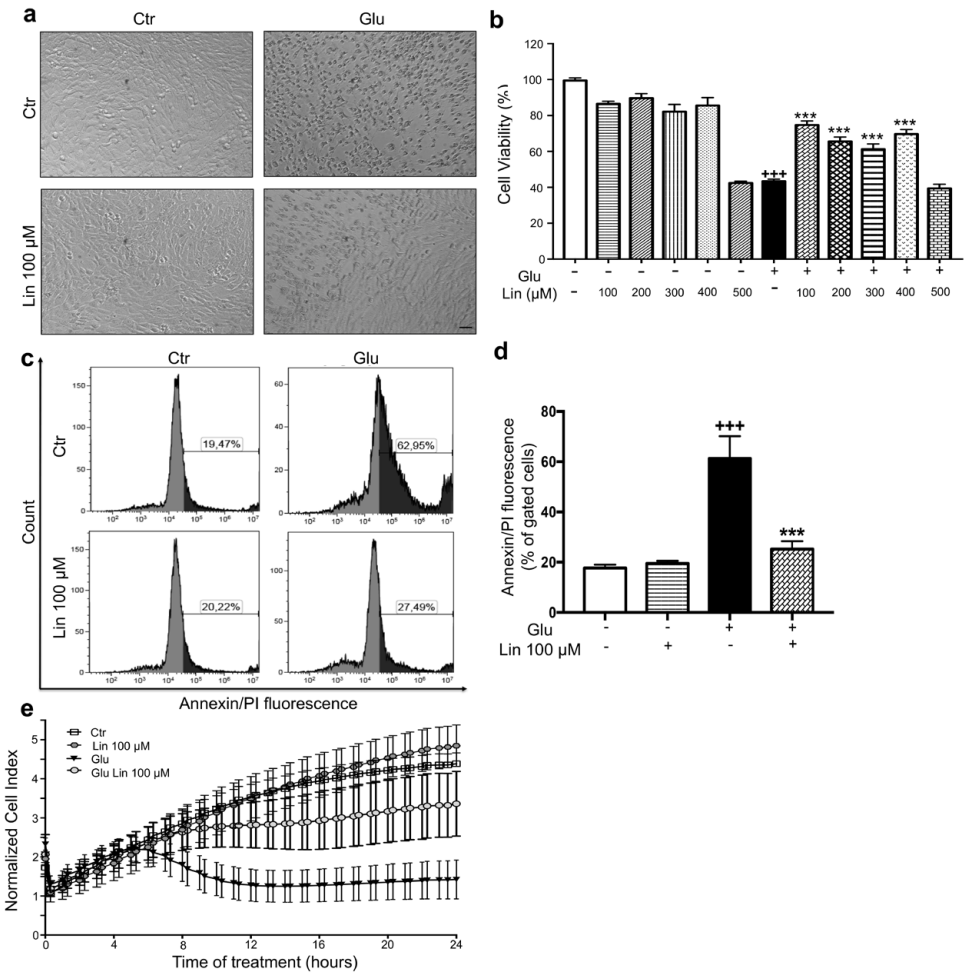


Fig. 1. Protective effects of linalool against glutamate in HT-22 cells. (a). Representative pictures of the cell morphology of HT-22 cells treated with glutamate (6 mM, 16 h) with and without linalool (100 µM) co-treatment. Magnification 10x (Scale bar: 100 µm) (b). Representative MTT assay of cells treated with glutamate and linalool in a range of 100 µM to 500 µM (n = 6 technical replicates; independent experiments repeated at least 3 times). (c,d). Measurement of the green Annexin V and the red PI fluorescence by FACS from HT-22 cells challenged with glutamate (6 mM) for 18 h in the absence and presence of linalool co-treatment (10,000 cells per condition; n = 3 technical replicates; +++ p < 0.001: untreated control versus glutamate, ***p < 0.001: glutamate versus linalool+glutamate-treated cells ANOVA, Tukey's test, independent experiments repeated at least 3 times). (e). Impedance-based real time detection of the morphological alterations of glutamate-treated HT-22 cells with and without linalool co-treatment (mean ± SD; n = 6 technical replicates). All experiments were repeated at least three times.

with or without linalool for 18 h. As shown in Fig. 1.c and d, the number of double positive stained cells was increased after glutamate exposure, indicating cell death, and this effect was largely attenuated by linalool application.

The protective effect of linalool against glutamate toxicity in HT-22 cells was also confirmed by real-time cell impedance measurements using the xCELLigence system. Cell impedance

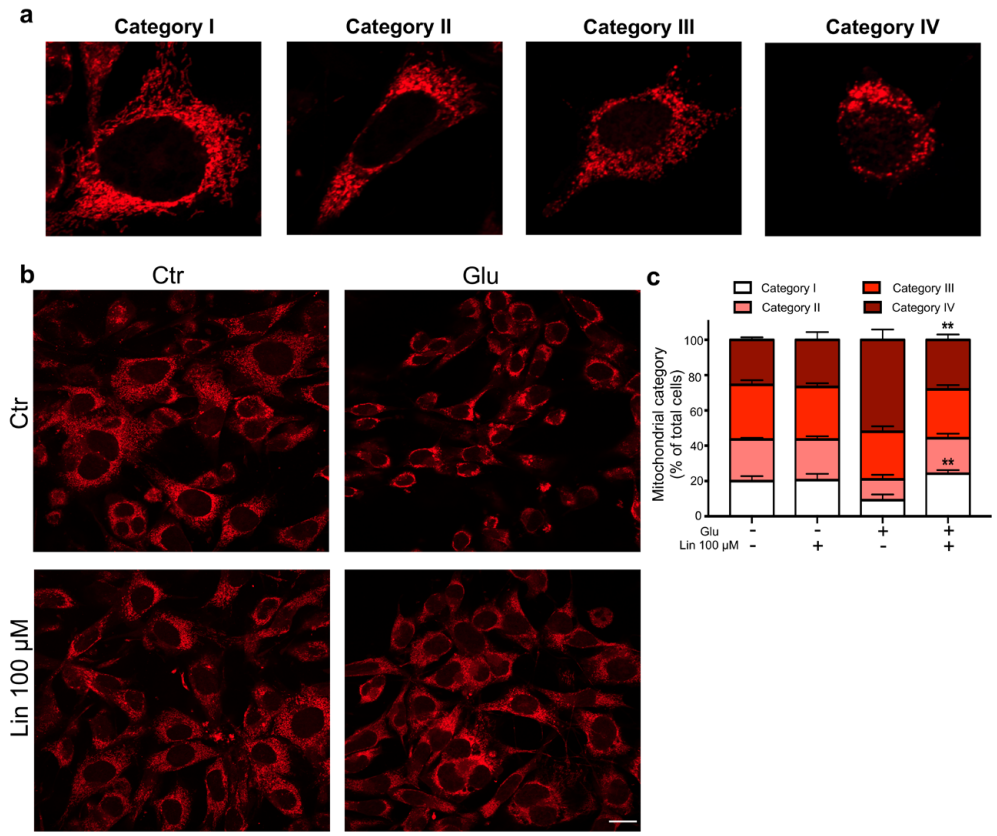


Fig. 2. Linalool attenuates mitochondrial fragmentation in glutamate-exposed HT-22 cells. (a). Mitochondrial morphology was classified into 4 categories of fragmentation (category I: mitochondria tubulin-like distributed equally throughout the cytosol; category II: short mitochondrial but still forming long tubules; category III: Large round mitochondria that begin to disrupt; category VI: Small rounded mitochondria of different sizes within proximity of the nucleus. (b). Changes of mitochondrial morphology were visualized following glutamate exposition -challenge (18 h – 6 mM) using MitoTracker Red (scale bar: 20 µm). (c). Quantification of mitochondrial categories in all conditions. 500 cells per condition; n = 3 independent experiments; mean ± SEM, **p < 0.01: glutamate versus linalool+ glutamate-treated cells ANOVA, Tukey's test.

measurements demonstrated that glutamate induces a strong decrease in cell index, an indication of cell detachment and cell death, approximately 10–14 h following the initiation of the glutamate treatment (Fig. 1.e). Co-treatment with linalool significantly delayed cell death, determined by a right shift of the cell impedance curves. Treatment with linalool alone did not alter the cell index or morphological shape of the cells. Interestingly, linalool did not prevent cell death when cells were challenged with erastin instead of glutamate (Figure suppl 1.b). Erastin was shown to induce ferroptosis cell death and associated with mitochondrial dysfunction and lipid peroxide formation through iron-dependent oxidative enzymes [10]. Taken together, these results show that linalool is able to significantly reduce glutamate-induced cell death in neuronal HT-22 cells.

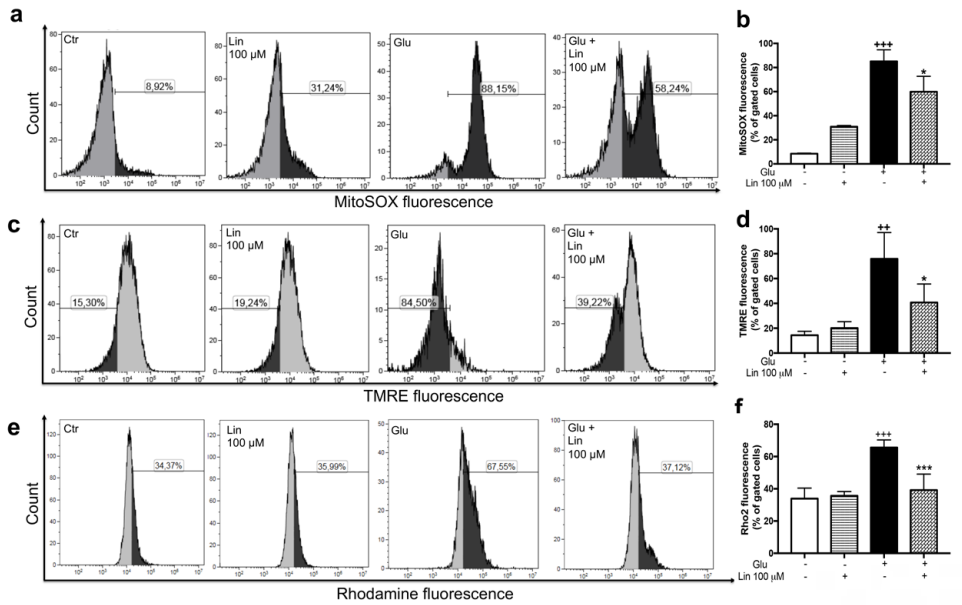


Fig. 3. Linalool preserved mitochondrial integrity. Mitochondrial integrity was analyzed by means of fluorescent-activated cell sorting using fluorescent probes for (a–b). mitochondrial ROS levels (MitoSOX), (c–d) mitochondrial membrane potential (TMRE) or (e–f) mitochondrial calcium (Rhod2 AM) following glutamate exposure (18 h) in the presence or absence of linalool 100 µM in HT-22 cells. Data are presented as mean ± SEM, n = 3–4 technical replicates, independent experiments repeated at least 3 times, *p < 0.05, **p < 0.01, ***p < 0.001: glutamate versus linalool + glutamate-treated cells ANOVA, Tukey's test.

3.2. Linalool preserves mitochondrial integrity in glutamate-treated HT-22 cells

The cell death pathway of oxytosis involves mitochondrial fragmentation and dysfunction. Since mitochondrial fragmentation could be initiated by glutamate [30], we assessed whether linalool could preserve mitochondrial morphology. First, we identified changes in mitochondrial morphology after glutamate exposure compared to control cells (Fig. 2.a–b). In untreated control cells, mitochondria show an elongated and tubular-like shape distributed along the cytosol. This mitochondrial shape is considered as category I and is most frequently found in healthy cells; while category II mitochondrial networks are less extensive than category I mitochondria but are still forming long tubules. However, in cells treated with glutamate, mitochondrial fission and fragmentation was elevated and category III and IV are more abundant. Category III is characterized by round mitochondria spread throughout the cell and category IV exhibit round and disintegrated mitochondria surrounding the nucleus. Examination of the acquired confocal images and their categorization (Fig. 2.a–b) indicated a significant difference between glutamate-treated cells and the remaining conditions, explicitly pertaining to mitochondrial categorization. HT-22 cells exposed to toxic glutamate showed a significant reduction in mitochondria of categories I and II while mitochondria of category IV was significantly increased. In accordance with the cell viability data, co-treatment of glutamate with linalool showed similar mitochondrial categories as the control healthy cells, indicating that linalool is able to preserve mitochondrial shape and prevent its fragmentation.

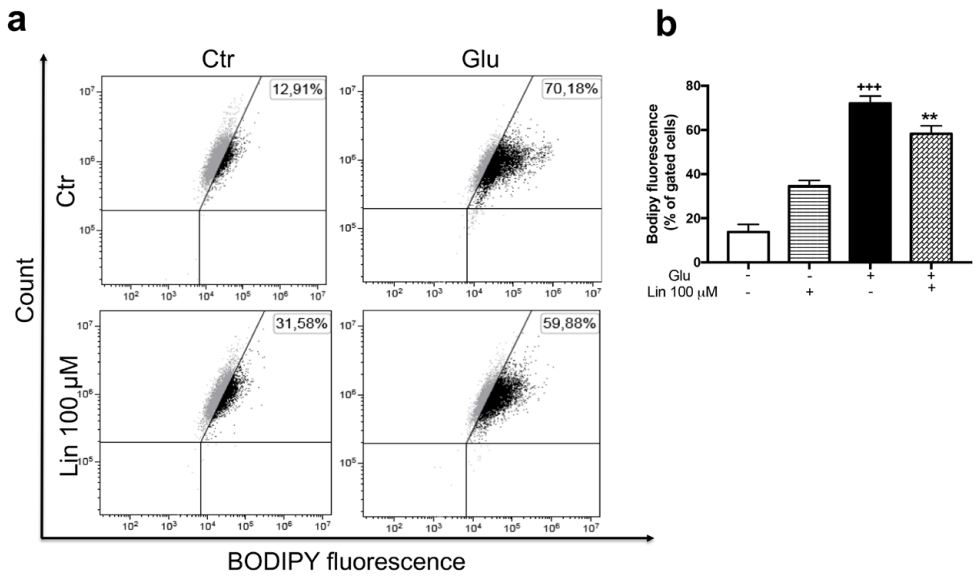


Fig. 4. Lipid peroxidation decreases with exposure of linalool after glutamate treatment. (a). Representative measurements of lipid peroxidation following glutamate exposure (18 h) in the presence or absence of linalool (Lin) in HT-22 cells. b. Bar graphs with statistics. Results are shown as mean ± SEM, n = 3 technical replicates. All experiments were repeated at least three times. **p < 0.01: glutamate versus linalool + glutamate-treated cells ANOVA, Tukey's test.

Under physiological conditions, mitochondria represent the major endogenous source of ROS as a byproduct of the electron transfer system (ETS). ROS generation is enhanced upon mitochondrial dysfunction, subsequently leading to oxidative damage to lipids, proteins, and DNA [34]. We evaluated the mitochondrial ROS levels using flow cytometry of the fluorescent MitoSOX dye, following glutamate exposure in the presence or absence of linalool. Analysis of the FACS data indicated that HT-22 cells stimulated with glutamate yielded a significant increase in mitochondrial ROS levels when compared to vehicle-treated cells. HT-22 cells co-treated with glutamate and linalool showed significantly decreased amounts of mitochondrial ROS levels when compared to the glutamate condition (Fig. 3.a–b).

Next, we evaluated the mitochondrial membrane potential (△ψm) by FACS analysis of the TMRE fluorescence, a dye specific for △ψm measurements. Our results indicated that glutamate challenge provoked a significant decrease in △ψm. Linalool alone did not result in a noticeable difference compared to vehicle-treated cells (Fig. 3.c–d), while in the presence of glutamate, it significantly alleviated the loss of △ψm.

Mitochondria play an essential role in buffering the cytosolic calcium overload in stimulated neurons. Therefore, we evaluated Rhod2AM fluorescence, specific for assessing mitochondrial Ca^{2+} levels by FACS measurements. Following glutamate treatment, we

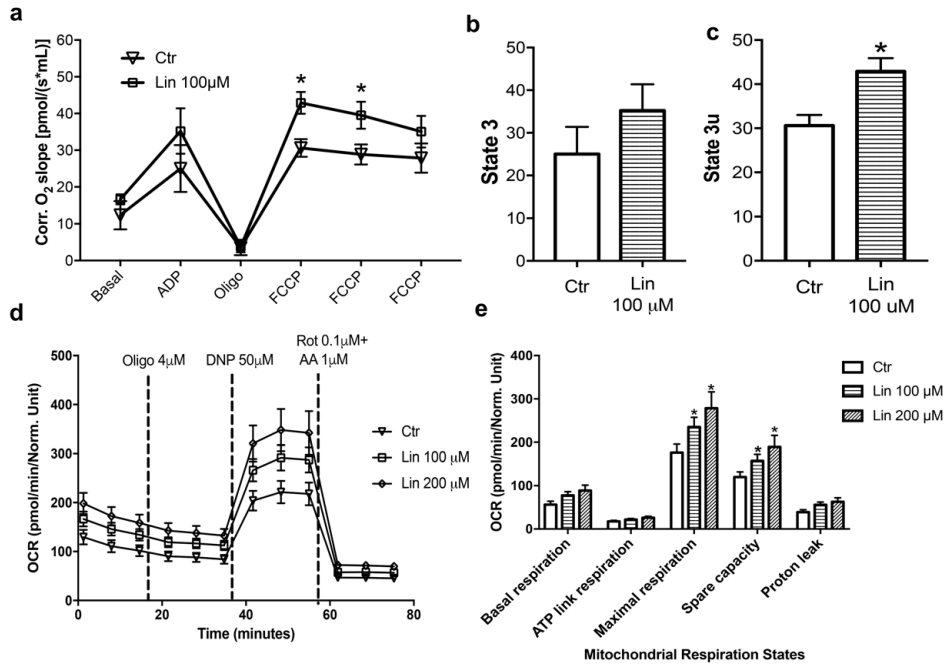


Fig. 5. Addition of linalool increases the uncoupled respiration in HT-22 cells. (a). Representative oxygen slope of isolated mitochondria from HT-22 cells. Corrected oxygen slope showing mitochondrial states after treatment with control (ethanol) or linalool 100 µM. (b–c). Quantification of state 3 and state 3 u of mitochondria pre-treated with control or linalool 100 µM in HT-22 cells (n = 3 technical replicates; independent experiments repeated at least 3 times). (d). Oxygen Consumption Rate (OCR) measurements in HT-22 cells treated with or without linalool 100 and 200 µM were obtained over time (min) using an extracellular flux analyzer (Seahorse Bioscience). The mitochondrial stress test was used to obtain bioenergetics parameters, by adding substrates (e). Maximal OCR was stimulated by DNP 50 u M addition and the spare respiratory capacity was calculated as the difference between maximal and basal OCR. Graphs show the OCR in pmoles per min. Data are shown as mean ± SEM, n = 3–6 technical replicates. All experiments were repeated at least three times. *p < 0.05 compared to indicated control.

observed an increase in the Rhod2AM fluorescence intensities, while co-treatment with linalool reduced the increase in mitochondrial Ca^{2+} levels (Fig. 3.e–f).

3.3. Linalool reduces lipid peroxidation in HT-22 cells

In HT-22 cells, glutamate exposure triggers an increase of detrimental ROS levels and also of lipid peroxides. To investigate the influence of linalool on lipid peroxide levels under oxidative stress, HT-22 cells were challenged with glutamate in the presence or absence of linalool for 18 h. Lipid peroxidation was examined by FACS analysis using BODIPY fluorescence. Exposure of HT-22 cells to glutamate induced a significant increase in lipid peroxidation, while linalool co-treatment was able to reduce the lipid peroxidation. Additionally, treatment with linalool-only was unable to produce a significant increase of this marker (Fig. 4.a–b). Taken together, linalool application enhanced protection against glutamate-induced oxidative toxicity by preserving mitochondrial morphology and reducing the detrimental levels of mitochondrial ROS, lipid peroxides, and mitochondrial Ca^{2+}.

3.4. Linalool increases mitochondrial respiration in HT-22 cells

Oxidative phosphorylation is a central energy-conserving mechanism, coupling mitochondrial electron transfer to ATP synthesis and providing the energy required for the maintenance of cellular functions and cell survival [35]. Assessment of mitochondrial respiration represents a functional evaluation of mitochondrial homeostatic state [36,37]. In the next step, to study alterations of the main mitochondrial metabolic functions, we performed high-resolution respirometry experiments in purified mitochondria of HT-22 cells. Interestingly, we found that linalool application to isolated mitochondria for a period of 25 min showed a tendency towards improved complex I-linked respiration rate, induced by ADP (state 3) (Fig. 5.b). Analysis of OXPHOS in isolated mitochondria from HT-22 revealed that linalool also promotes an increase in maximum uncoupled respiration induced by FCCP (Fig. 5.a.b).

To complete the characterization of the respiratory capacity of the mitochondria (depicted as oxygen consumption rate; OCR), we used Seahorse Bioscience XF96 Extracellular Flux Analyzer. Fig. 5.d.e. showed an increase in the mitochondrial respiration states, particularly, in the maximal respiration values as well as the spare respiratory capacity with the addition of linalool 100 µM to HT-22 cells, when compared with solvent-treated control cells. This increase in values is concentration dependent, as linalool 200 µM elicited a higher increase in respiration levels compared to linalool 100 µM. Considering these results, linalool improved mitochondrial respiration when compared to vehicle-treated HT-22 cells and also in isolated mitochondria.

3.5. Linalool significantly reduced NMDA-mediated cell death and cyclooxygenase-2 in organotypic brain slices

In order to corroborate the *in vitro* data (HT-22 cell line), potential neuroprotection effects of linalool were also evaluated *ex vivo* in OHSC. This *ex vivo* model is a good method for assessing neuronal death, microglial activation, neurogenesis, and drug screening. OHSC mainly preserves tissue structures, maintain neuronal activities and synapse circuitry, and mimic many aspects of the *in vivo* context [38]. After six days in culture, we exposed OHSC to 10 µM NMDA in the presence or absence of linalool for 24 h (Fig. 6.a). Cell death was measured by Propidium Iodide (PI) uptake using imaging quantification of the hippocampal slices. NMDA stimuli induced a strong increase in PI uptake in the CA1 and GD regions of the hippocampus, while the CA3 region of the hippocampus was less affected. Co-treatment with linalool significantly reduced the PI uptake in CA1 hippocampal region, although the NMDA-induced damage of GD and CA3 regions was less affected by linalool application (Fig. 6.b.c). Concerning apoptosis evaluation, hippocampal slices challenged with NMDA stimuli showed an increase, although not significant to apoptotic Bax protein expression (Fig. 6.d).

To determine if any anti-/pro-inflammatory genes of the signaling systems were involved in the protective effect of linalool on NMDA-induced excitotoxicity in the hippocampus, we analyzed the expression of the following genes: Interleukin 1 beta (*Il-1ß*), Nitric Oxide

Linalool attenuates oxidative stress and mitochondrial dysfunction mediated by glutamate damage

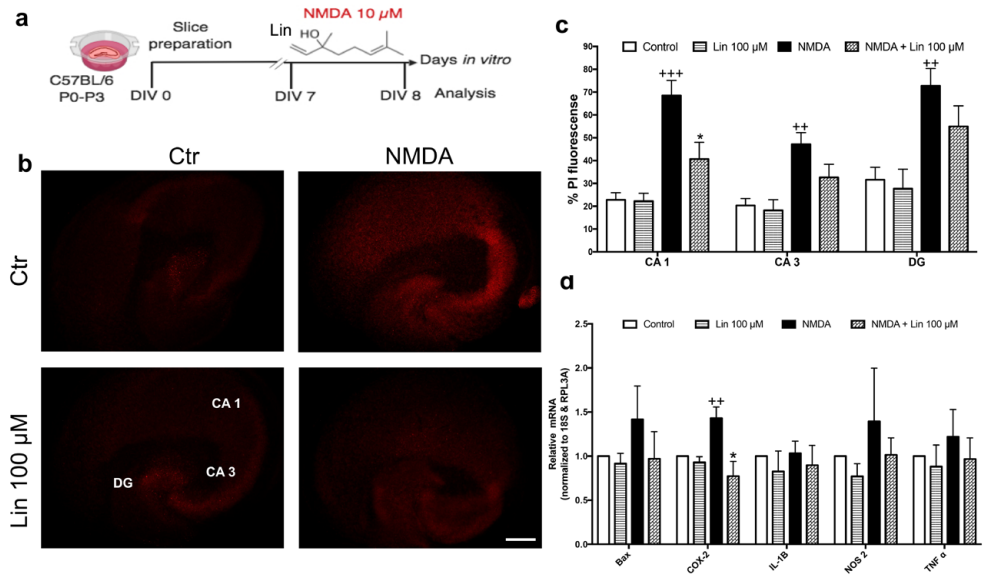


Fig. 6. The effect of linalool on NMDA excitotoxicity in mouse OHSCs. (a). Experimental time schedule for treatment with linalool after 10 µM NMDA challenge for 24 h. (b). PI staining images indicating the effects of vehicle, linalool and NMDA alone and NMDA + linalool on hippocampal cell damage. Magnification 5x (Scale bar: 50 µm) (c). PI fluorescence into CA1, CA3 and DG areas of each sample was expressed as % of total PI fluorescence. (d). qPCR analysis of different treatments in slice cultures revealed differences in the levels of Cox-2 in the NMDA treatment compared with the control. No differences were observed in Bax, Il-1β, Nos2 and Tnf-α. Data are shown as mean ± SEM n = 4–6 independent experiments). + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$: untreated control vs NMDA, *$p < 0.05$: NMDA versus linalool+NMDA-treated OHSC ANOVA, Tukey's test.

Synthase 2 (*Nos2*), Tumor necrosis factor alpha (*Tnfα*) and cyclooxygenase-2 (*Cox-2*) genes by RT-qPCR. *Cox-2* gene expression was increased after NMDA treatment and treatment with linalool reversed this proinflammatory marker to levels similar to the controls (Fig. 6.d). *Nos2* gene expression seems to increase, albeit no significant values were detected following NMDA treatment, while co-treatment of linalool showed a tendency to decrease the levels of *Nos2* genes. *Il-1ß* and *Tnfα* did not show any differences between the treatments (Fig. 6.d). However, NMDA challenge of OHSC did significantly increase Cox-2 levels when compared to OHSC-vehicle conditions (post hoc $p < 0.01$). Data obtained in OHSC cultures validated the neuroprotective effects of linalool detected in neuronal HT-22 cell line. Furthermore, our results reinforce the hypothesis where the mitochondria are involved in the mechanism of protection when is activated by this monoterpene.

4. Discussion

Our findings demonstrate a neuroprotective effect of linalool in conditions of oxidative stress through mechanisms involving an increase in mitochondrial respiration, and preservation of several mitochondrial parameters, including ROS, calcium levels, and $\triangle\psi m$. Furthermore,

linalool mediated protection against NMDA-induced excitotoxicity in hippocampal slices. Taken together, we showed that linalool induced neuroprotective effects in an *in vitro* and *ex vivo* models by preserving and restoring mitochondrial function.

In this study, we showed that linalool improves the viability of neuronal cells by 30% in conditions of oxidative stress, independent of excitotoxicity. The *in vitro* model of oxytosis was employed in neuronal HT-22 cells, which lack the NMDAR, therefore, glutamate stimulation initiates a distinct pathway of cell death independent of NMDA-related increased neuronal excitability [5]. In this paper, we determined the potential timeframe of linalool's protection, which was detected following 10 h of glutamate challenge and was maintained longer than 24 h. Our results are in line with findings investigating the effects of linalool in glucose/serum deprivation (GSD)-induced cytotoxicity. Under GSD conditions, linalool was able to exert neuroprotective effects following 8 h of GSD in PC12 cells [16]. Similarly, in neuronal cultures has been shown that after oxygen-glucose deprivation/reoxygenation (OGD/R) or glutamate-mediated excitotoxity as *in vitro* model of ischemic stroke, linalool attenuated cell death. The neuroprotection was mediated by scavenging peroxyl-radicals, increasing SOD and catalase levels. We have previously demonstrated the role of linalool *in vitro* in a glutamate excitotoxicity model where linalool restored intracellular ATP levels in neurons and astrocytes [28]. Our results parallel another research using a different monoterpene, catalpol, that was shown to exert neuroprotection in the hippocampal CA1 area of a gerbil transient global cerebral ischemia model [39].

Mitochondrial fusion and fission processes are tightly coordinated by several dynamin protein family members (e.g. fission 1, dynamin related protein 1, endophilin B1), in response to environmental changes and cellular stress [30,40]. Based on previous research which showed that glutamate induces mitochondrial damage in HT-22 cells [2,41], we investigated whether linalool was capable of moderating this effect. Our results showed that linalool HT-22 cells prevent damaged or dying cells (mitochondrial category IV) stimulated with glutamate. Further, linalool prevented mitochondrial fragmentation and peri-nuclear accumulation of the organelles providing further evidence of its beneficial impact on mitochondria. Besides, we investigated the ability of linalool to modify the mitochondrial membrane potential ($\triangle\psi m$). The latter is a consequence of the proton gradient which exists across the inner mitochondrial membrane and is utilized for ADP phosphorylation. The net negative charge across a healthy mitochondrion is essentially generated by cytochrome c, which shuttles electrons along the ETS. How-ever, this function is impaired during oxytosis which not only leads to mitochondrial membrane permeabilization but also to release cytochrome c into the cytosol leading to immediate dissipation of $\triangle\psi m$ [5]. Our findings showed that linalool enhanced mitochondrial respiration and preserved $\triangle\psi m$ which might be a consequence of the conserved inner mitochondrial membrane (IMM).

The brain is particularly susceptible to oxidative stress due to its high content of unsaturated phospholipids [42]. Oxidative stress is highly associated with pathological conditions such as AD, in relation to Aβ plaque burden, and cerebral ischemia, initiated by excessive release of excitatory neurotransmitters and Ca^{2+} leading to increased ROS formation [43,44]. Our results showed co-treatment of glutamate-stimulated HT-22 cells with linalool leads to a reduction of ROS levels. Previous studies showed that linalool reduced intracellular oxidative stress in the OGD/R condition [45]. Furthermore, linalool prevented glutamate-induced cell death alongside retraction of processes and actin cytoskeleton depolymerization in neurons [28]. However, glutamate-stimulation in HT-22 cells yields a well-defined program of cell death via oxytosis; therefore, our findings suggest that the reduction in ROS levels might be due to an increase of intracellular GSH availability. The latter is in line with previous research that showed linalool reduced oxidative stress and the levels of malondialdehyde in an animal model of neurotoxicity produced by acrylamide increasing the GSH levels [46].

We reported for the first time, the effect of linalool on mitochondrial respiration in a hippocampal cell line. Mitochondrial respiration is the power source of eukaryotic cells due to provision of ATP by means of OXPHOS [47]. We observed an increase in maximal oxygen consumption with linalool. Maximal oxygen consumption rate attained by adding the uncoupler FCCP. FCCP mimics a physiological "energy de-mand" by stimulating the respiratory chain to operate at maximum capacity, which causes rapid oxidation of substrates to meet this metabolic challenge [48]. This indicates that increased maximal respiration and spare capacity is associated with increased metabolic fitness as it makes the cell better able to cope with stress. There are few studies about monoterpenes involved in complex of mitochondrial respiration. It has been described by Usta et al., 2009 that linalool reduced cell viability of cancer cells (HepG2) by inhibition of complexes I and II and decreasing ATP [49]. Other monoterpenes as β-Pinene showed uncoupling effects at lower concentrations (100–200 μM) than the inhibition of respiration (400 μM) through an effect on the electron transport chain in the liver of Wistar rats [50]. In addition, a combination of monoterpenes camphene and geraniol (1:1) protected against nimesulide hepatotoxicity *in vivo*, reducing the mitochondrial swelling, inhibition in release of apoptotic proteins and prevented mitochondrial depolarization along with reduction in oxidized NAD(P)H and increased mitochondrial electron flow [51]. These findings demonstrated that monoterpenes have a potential to regulate mitochondrial respiration in different disease models.

To validate *in vitro* data, we evaluated the effects of linalool in OHSC. We observed an increase in cell death on response to NMDA. Linalool was found to protect areas in the hippocampus such as CA1, however, we did not detect significant differences between other regions of hippocampus due to the variability of cell damage. Additionally, we evaluated genes involved in inflammation and cell death which seemed to increase in response to NMDA. *Cox-2* is usually expressed at low levels under normal conditions, but is rapidly

induced by an injury, triggers pro-inflammatory response with prostaglandins and can worsen neuronal degeneration in neurological diseases such as stroke [52,53]. We detected a significant increase in *Cox-2* gene expression after NMDA treatment while linalool reduced this increase. In previous research, we observed a reduction in the levels of inflammatory markers such as p38 MAPK and *Cox-2* after linalool treatment for 3 months in aged 3xTg-AD mice [27]. Similarly, reduction of Cox-2 levels was also observed post-linalool treatment for 1 month in a global ischemic model [28]. This result is further supported by other studies such as reduced levels of cyclooxygenases in other neurotoxicity models when stimulated with linalool and decrease in a dose-dependent manner in LPS-stimulated macrophages [54,55].

Recent studies have shown that cytokines such as TNF-α, IL-1β, IL-1α, IFN-γ are synthesized in the CNS and play an important role in inflammation. This release is promoted after glutamate excitotoxicity [17]. Linalool administered to mice inhibits behavioral nociceptive responses caused by proinflammatory cytokines Il-1β and TNF-α via inhibition of NMDA receptors [17,25]. However, we did not find any significant increase of these proinflammatory cytokines (NO2, IL-1β, TNF-α) due to variability in the cell damage in the hippocampus.

We have previously described the protective effects of linalool in a 3xTg-AD mice [27] and in cerebral ischemia [28]. Besides, linalool protects against acrylamide (ACR)-induced neurotoxicity in Wistar rats decreasing the ACR-induced lipid peroxidation in rat brain tissue [46]. Linalool protection can by mediated by the regulation of glutamatergic system [56,57], and axonal regeneration [58], among other properties described in mentioned literature.

5. Conclusion

Our results illustrate that linalool positively impacts the mitochondrial functioning of glutamate-stimulated HT-22 cells. We observed linalool reduced ROS, calcium production, and lipid peroxidation levels. Additionally, improved mitochondrial morphology, membrane potential, and respiration. This together results in an increase of cell viability. Importantly, this study is among the first to elucidate the particular underlying mechanism by which linalool exerts its effectiveness, precisely via a protective impact on the mitochondria. We validate the *in vitro* neuroprotective data with *ex vivo* OHSC slices where we showed a reduction of cell death in the hippocampus and modulation of various genes by linalool. Our data indicate that linalool may be therapeutically useful in treating neurodegenerative conditions marked by mitochondrial dysfunction and subsequent ROS production, due to its beneficial impact on mitochondrial integrity as well as ability to limit oxidative stress.

Linalool attenuates oxidative stress and mitochondrial dysfunction mediated by glutamate damage

Author contribution

A.M.S.G designed and realized the experiments, analyzed the data and wrote the paper; F.H help in high-resolution respirometry experiments; A.K performed the quantification of categories of mitochondria; A.O performed Seahorse experiments; E.B and A.K. interpreted data and review the manuscript; A.M.D. designed, analyzed and interpreted the data, prepared the manuscript and provided critical revision. All authors read and approved the final manuscript.

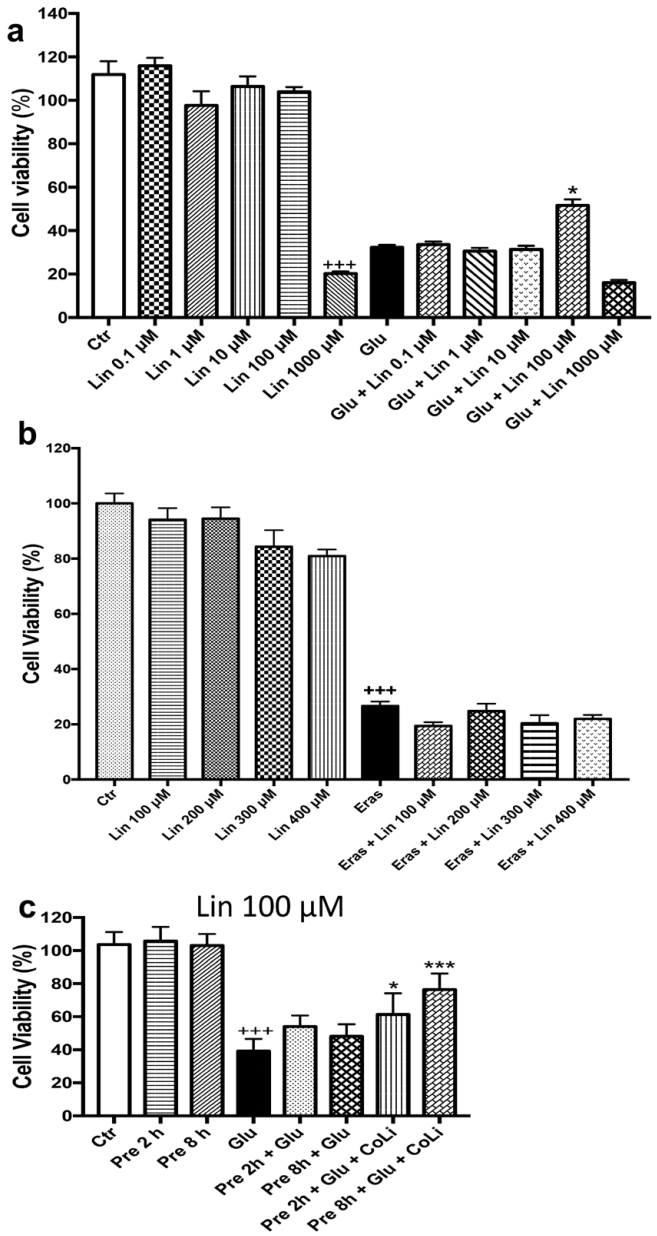
Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank the Molecular Pharmacology group of University of Groningen for providing scientific and technical support during the experiments. In particular, we thank Inge Krabbendam for support on the cell culture and FACs mitochondrial analysis. In addition, we acknowledge Albert Gerding and Prof. Barbara Bakker from UMCG for their support on high-respirometry experiments and Professor Patricia Cardona from University of Antioquia for her advice during the project. This research was supported by Scholarship Colciencias call 647 (AMS-G) and Abel Tasman program. A.M.D. is the recipient of a Rosalind Franklin Fellowship co-funded by the European Union and the University of Groningen.

Supplementary figure



Supplementary Figure 1. Linalool inhibits glutamate-induced oxytosis but not erastin-induced cell death. Cell viability was evaluated in HT-22 cells with an MTT assay. (a). Cells were co-treated with glutamate 6mM and Linalool in a range of 0.1, 1.0, 10, 100, 1000 µM. (b). Cells were treated with erastin 1.5 µM and Linalool in a range of 100 to 400 µM. All experiments were repeated at least three times with 5-6 technical replicates. (+++p < 0.001: non-treated cells vs glutamate or erastin; *p < 0.05: glutamate or erastin versus cells treated with different concentrations of linalool - ANOVA, Tukey test).

References

[1] S. Neitemeier, A.M. Dolga, B. Honrath, S.S. Karuppagounder, I. Alim, R.R. Ratan, C. Culmsee, Inhibition of HIF-prolyl-4-hydroxylases prevents mitochondrial impairment and cell death in a model of neuronal oxytosis, Cell Death Diseas. 7 (2016) e2214–13, https://doi.org/10.1038/cddis.2016.107.

[2] A.M. Dolga, M.F. Netter, F. Perocchi, N. Doti, L. Meissner, S. Tobaben, J. Grohm, H. Zischka, N. Plesnila, N. Decher, C. Culmsee, Mitochondrial small conductance SK2 channels prevent glutamate-induced oxytosis and mitochondrial dysfunction, J. Biol. Chem. 288 (2013) 10792–10804, https://doi.org/10.1074/jbc.M113.453522.

[3] S. Tan, D. Schubert, P. Maher, Oxytosis: a novel form of programmed cell death, Curr. Top. Med. Chem. 1 (2001) 497–506, https://doi.org/10.2174/1568026013394741.

[4] J. Lewerenz, G. Ates, A. Methner, M. Conrad, P. Maher, Oxytosis/ferroptosis-(Re-) emerging roles for oxidative stress-dependent non-apoptotic cell death in diseases of the central nervous system, Front. Neurosci. 12 (2018), https://doi.org/10.3389/ fnins.2018.00214.

[5] A.M. Dolga, S. Oppermann, M. Richter, B. Honrath, S. Neitemeier, A. Jelinek, G. Ganjam, C. Culmsee, Molecular mechanisms underlying oxytosis, Apoptosis Beyond Many Ways Cells Die, (2018), pp. 289–316, https://doi.org/10.1002/ 9781119432463.ch14.

[6] W. Li, S. Yang, Targeting oxidative stress for the treatment of ischemic stroke: upstream and downstream therapeutic strategies, Brain Circ. 2 (2016) 153, https:// doi.org/10.4103/2394-8108.195279.

[7] P. Li, R.A. Stetler, R.K. Leak, Y. Shi, Y. Li, W. Yu, M.V.L. Bennett, J. Chen, Oxidative stress and DNA damage after cerebral ischemia: potential therapeutic targets to repair the genome and improve stroke recovery, Neuropharmacology 134 (2018) 208–217, https://doi.org/10.1016/j.neuropharm.2017.11.011.

[8] P. Maher, Free Radical Biology and Medicine Potentiation of glutathione loss and nerve cell death by the transition metals iron and copper: implications for age-related neurodegenerative diseases, Free Radic. Biol. Med. 115 (2018) 92–104, https://doi.org/10.1016/j.freeradbiomed.2017.11.015.

[9] T.H. Murphy, M. Miyamoto, A. Sastre, R.L. Schnaar, J.T. Coyle, Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress, Neuron 2 (1989) 1547–1558, https://doi.org/10.1016/0896-6273(89) 90043-3.

[10] S.J. Dixon, K.M. Lemberg, M.R. Lamprecht, R. Skouta, M. Eleina, C.E. Gleason, D.N. Patel, A.J. Bauer, A.M. Cantley, W.S. Yang, B.M. Iii, B.R. Stockwell, Ferroptosis: an iron-dependent form of non-apoptotic cell death, Cell 149 (2012) 1060–1072, https://doi.org/10.1016/j.cell.2012.03.042.Ferroptosis.

[11] S. Neitemeier, A. Jelinek, V. Laino, L. Hoffmann, I. Eisenbach, R. Eying, G.K. Ganjam, A.M. Dolga, S. Oppermann, C. Culmsee, BID links ferroptosis to mitochondrial cell death pathways, Redox. Biol. 12 (2017) 558–570, https://doi.org/ Biomedicine & Pharmacotherapy 118 (2019) 109295 3382/ps.2013-03254.

[12] P. Maher, The effects of stress and aging on glutathione metabolism, Ageing Res. Rev. 4 (2005) 288–314, https://doi.org/10.1016/j.arr.2005.02.005.

[13] A.A. Kritis, E.G. Stamoula, K.A. Paniskaki, T.D. Vavilis, Researching glutamate –induced cytotoxicity in different cell lines: a comparative/ collective analysis/ study, Front. Cell. Neurosci. 9 (2015) 1–18, https://doi.org/10.3389/fncel.2015. 00091.

[14] K.P. Volcho, S.S. Laev, G.M. Ashraf, G. Aliev, N.F. Salakhutdinov, Application of monoterpenoids and their derivatives for treatment of neurodegenerative disorders, Curr. Med. Chem. 25 (2018) 5327–5346, https://doi.org/10.2174/0929867324666170112101837.

[15] X. Wang, G. Li, W.E.I. Shen, Protective effects of D–Limonene against transient cerebral ischemia in stroke–prone spontaneously hypertensive rats, Exp. Ther. Med. 15 (2018) 699–706, https://doi.org/10.3892/etm.2017.5509.

[16] B. Alinejad, A. Ghorbani, H.R. Sadeghnia, Effects of combinations of curcumin, linalool, rutin, safranal, and thymoquinone on glucose/serum deprivation-induced cell death, Avicenna J. Phytomed. 3 (2013) 321, https://doi.org/10.22038/AJP. 2013.418.

[17] P.A. Batista, M.F. De Paula Werner, E.C. Oliveira, L. Burgos, P. Pereira, L.F. Da Silva Brum, G.M. Story, A.R.S. Santos, The antinociceptive effect of (-)-linalool in models of chronic inflammatory and neuropathic hypersensitivity in mice, J. Pain 11 (2010) 1222–1229, https://doi.org/10.1016/j.jpain.2010.02.022.

[18] E. Elisabetsky, J. Marschner, D. Onofre Souza, Effects of linalool on glutamatergic system in the rat cerebral cortex, Neurochem. Res. 20 (1995) 461–465, https://doi. org/10.1007/BF00973103.

[19] V.M. Linck, A.L. da Silva, M. Figueiró, E.B. Caramão, P.R.H. Moreno, E. Elisabetsky, Effects of inhaled Linalool in anxiety, social interaction and aggressive behavior in mice, Phytomedicine 17 (2010) 679–683, https://doi.org/10.1016/j.phymed.2009.

[20] P.J.C. Anjos, A.O. Lima, P.S. Cunha, D.P. De Sousa, A.S.C. Onofre, T.P. Ribeiro, I.A. Medeiros, Â. Antoniolli,

L.J. Quintans-Júnior, M.R.V. Santos, Cardiovascular effects induced by linalool in normotensive and hypertensive rats, Zeitschrift Für Naturforsch. C 68 (2013) 181–190, https://doi.org/10.1515/znc-2013-5-603.

[21] R.C. Beier, J.A.B. Ii, L.F. Kubena, M.E. Hume, J.L. Mcreynolds, R.C. Anderson, D.J. Nisbet, Evaluation of linalool, a natural antimicrobial and insecticidal essential oil from basil: Effects on poultry, Poult. Sci. 93 (2014) 267–272, https://doi.org/10.3382/ps.2013-03254.

[22] S.Y. Cho, H.J. Jun, J.H. Lee, Y. Jia, K.H. Kim, S.J. Lee, Linalool reduces the expression of 3-hydroxy-3-methylglutaryl CoA reductase via sterol regulatory element binding protein-2- and ubiquitin-dependent mechanisms, FEBS Lett. 585 (2011) 3289–3296, https://doi.org/10.1016/j.febslet.2011.09.012.

[23] V. Coelho, L. Mazzardo-Martins, D.F. Martins, A.R.S. Santos, L.F. da Silva Brum, J.N. Picada, P. Pereira, Neurobehavioral and genotoxic evaluation of (-)-linalool in mice, J. Nat. Med. 67 (2013) 876–880, https://doi.org/10.1007/s11418-013-0751-6.

[24] S.L. Guzmán-Gutiérrez, H. Bonilla-Jaime, R. Gómez-Cansino, R. Reyes-Chilpa, Linalool and β-pinene exert their antidepressant-like activity through the monoaminergic pathway, Life Sci. 128 (2015) 24–29, https://doi.org/10.1016/j.lfs.2015. 02.021.

[25] Q. Wu, L. Yu, J. Qiu, B. Shen, D. Wang, L.W. Soromou, H. Feng, Linalool attenuates lung inflammation induced by Pasteurella multocida via activating Nrf-2 signaling pathway, Int. Immunopharmacol. 21 (2014) 456–463, https://doi.org/10.1016/j. intimp.2014.05.030.

[26] S. Celik, A. Ozkaya, Effects of intraperitoneally administered lipoic acid, vitamin E, and linalool on the level of total lipid and fatty acids in guinea pig brain with oxidative stress induced by H2O2 , J. Biochem. Mol. Biol. 35 (2002) 547–552, https://doi.org/10.5483/BMBRep.2002.35.6.547.

[27] A.M. Sabogal-Guáqueta, E. Osorio, G.P. Cardona-Gómez, Linalool reverses neuropathological and behavioral impairments in old triple transgenic Alzheimer's mice, Neuropharmacology 102 (2016) 111–120, https://doi.org/10.1016/j.neuropharm. 2015.11.002.

[28] A.M. Sabogal-Guaqueta, R. Posada-Duque, N.C. Cortes, J.D. Londoño-Arias, G.P. Cardona-Gomez, Neuropharmacology changes in the hippocampal and peripheral phospholipid profiles are associated with neurodegeneration hallmarks in a long-term global cerebral ischemia model: Attenuation by Linalool, Neuropharmacology 135 (2018) 555–571, https://doi.org/10.1016/j.neuropharm. 2018.04.015.

[29] C.M. Dowling, C.H. Ors, P.A. Kiely, Using real-time impedance-based assays to monitor the effects of fibroblast-derived media on the adhesion, proliferation, migration and invasion of colon cancer cells, Biosci. Rep. 34 (2014) 415–427, https:// doi.org/10.1042/BSR20140031.

[30] J. Grohm, N. Plesnila, C. Culmsee, Bid mediates fission, membrane permeabilization and peri-nuclear accumulation of mitochondria as a prerequisite for oxidative neuronal cell death, Brain Behav. Immun. 24 (2010) 831–838, https://doi.org/10. 1016/j.bbi.2009.11.015.

[31] B. Honrath, L. Matschke, T. Meyer, L. Magerhans, F. Perocchi, G.K. Ganjam, H. Zischka, C. Krasel, A. Gerding, B.M. Bakker, M. Bünemann, S. Strack, N. Decher, C. Culmsee, A.M. Dolga, SK2 channels regulate mitochondrial respiration and mitochondrial Ca2+ uptake, Cell Death Differ. (2017) 1–13, https://doi.org/10. 1038/cdd.2017.2.

[32] J. Vinet, H.R.J. Van Weering, A. Heinrich, R.E. Kälin, A. Wegner, N. Brouwer, F.L. Heppner, N. Van Rooijen, H.W. Boddeke, K. Biber, Neuroprotective function for ramified microglia in hippocampal excitotoxicity, J. Neuroinflammation 9 (2012) 1–15, https://doi.org/10.1186/1742-2094-9-27.

[33] J.M. Ruijter, M.W. Pfaffl, S. Zhao, A.N. Spiess, G. Boggy, J. Blom, R.G. Rutledge, 10.1016/j.redox.2017.03.007. [12] P. Maher, The effects of stress and aging on glutathione metabolism, Ageing Res. Rev. 4 (2005) 288–314, https://doi.org/10.1016/j.arr.2005.02.005.

[34] K.J. Barnham, C.L. Masters, A.I. Bush, Neurodegenerative diseases and oxidative stress, Nat. Rev. Drug Discov. 3 (2004) 205, https://doi.org/10.1038/nrd1330.

[35] C. Affourtit, H. Wong, M.D. Brand, Measurement of proton leak in isolated mitochondria, Mitochondrial Bioenerg. Methods Protoc. Methods Mol. Biol. (2018), pp. 157–170, https://doi.org/10.1007/978-1-4939-7831-1_9.

[36] G. Kroemer, L. Galluzzi, C. Brenner, Mitochondrial membrane permeabilization in cell death, Physiol. Rev. 87 (2007) 99–163, https://doi.org/10.1152/physrev.00013.2006.

[37] M. Picard, T. Taivassalo, G. Gouspillou, R.T. Hepple, Mitochondria: isolation, structure and function, J. Physiol. 589 (2011) 4413–4421, https://doi.org/10.1113/jphysiol.2011.212712.

[38] Q. Li, X. Han, J. Wang, Organotypic hippocampal slices as models for stroke and traumatic brain injury, Mol. Neurobiol. 53 (2016) 4226–4237, https://doi.org/10. 1007/s12035-015-9362-4.

[39] D.-Q. Li, Y. Li, Y. Liu, Y.-M. Bao, B. Hu, L.-J. An, Catalpol prevents the loss of CA1 hippocampal neurons and reduces working errors in gerbils after ischemia-reperfusion injury, Toxicon 46 (2005) 845–851, https://doi.org/10.1016/j.toxicon. 2004.09.007.

[40] M. Karbowski, S.-Y. Jeong, R.J. Youle, Endophilin B1 is required for the maintenance of mitochondrial morphology, J. Cell Biol. 166 (2004) 1027–1039, https://doi.org/10.1083/jcb.200407046.

[41] P. Maher, K.F. Salgado, J.A. Zivin, P.A. Lapchak, A novel approach to screening for new neuroprotective compounds for the treatment of stroke, Brain Res. 1173 (2007) 117–125, https://doi.org/10.1016/j.brainres.2007.07.061. 10.002.

[42] G. Paradies, G. Petrosillo, V. Paradies, F.M. Ruggiero, Mitochondrial dysfunction in brain aging: role of oxidative stress and cardiolipin, Neurochem. Int. 58 (2011) 447–457, https://doi.org/10.1016/j.neuint.2010.12.016.

[43] Á. Chamorro, U. Dirnagl, X. Urra, A.M. Planas, Neuroprotection in acute stroke: targeting excitotoxicity, oxidative and nitrosative stress, and inflammation, Lancet 15 (2016) 869–881, https://doi.org/10.1016/S1474-4422(16)00114-9.

[44] Z. Chen, C. Zhong, Oxidative stress in Alzheimer's disease, Neurosci. Bull. 30 (2014) 271–281, https://doi.org/10.1007/s12264-013-1423-y.

[45] H. Park, G.H. Seol, S. Ryu, I.-Y. Choi, Neuroprotective effects of (-)-linalool against oxygen-glucose deprivation-induced neuronal injury, Arch. Pharm. Res. 39 (2016) 555–564, https://doi.org/10.1007/s12272-016-0714-z.

[46] S. Mehri, M.A. Meshki, H. Hosseinzadeh, Linalool as a neuroprotective agent against acrylamide-induced neurotoxicity in Wistar rats, Drug Chem. Toxicol. 38 (2015) 162–166, https://doi.org/10.3109/01480545.2014.919585.

[47] J. Das, The role of mitochondrial respiration in physiological and evolutionary adaptation, Bioessays 28 (2006) 890–901, https://doi.org/10.1002/bies.20463.

[48] C. Doerrier, L.F. Garcia-souza, G. Krumschnabel, Y. Wohlfarter, A.F. Mészáros, E. Gnaiger, High-resolution fluoRespirometry and OXPHOS protocols for human cells, permeabilized fibers from small biopsies of muscle, and isolated mitochondria, Mitochondrial Bioenerg., Springer Protocols, (2018), pp. 31–70, https://doi. org/10.1007/978-1-4939-7831-1_3.

[49] J. Usta, S. Kreydiyyeh, K. Knio, P. Barnabe, Y. Boumoughlabay, S. Dagher, Linalool decreases HepG2 viability by inhibiting mitochondrial complexes I and II, increasing reactive oxygen species and decreasing ATP and GSH levels, Chem. Biol. Interact. 180 (2009) 39–46, https://doi.org/10.1016/j.cbi.2009.02.012.

[50] S. Uribe, R. Alvarez, A. Peña, Effects of b-pinene, a non substituted monoterpene, on rat liver mitochondria, Pestic. Biochem. Physiol. 50 (1984) 43–50, https://doi.org/ Disease, J. Neuroimmunol. 291 (2016) 115–124, https://doi.org/10.1016/j. jneuroim.2015.12.003.

[51] B.K. Singh, M. Tripathi, B.P. Chaudhari, P.K. Pandey, P. Kakkar, Natural terpenes prevent mitochondrial dysfunction, oxidative stress and release of apoptotic proteins during nimesulide-hepatotoxicity in rats, PLoS One 7 (2012) 1–11, https:// doi.org/10.1371/journal.pone.0034200.

[52] S. Sil, T. Ghosh, Role of cox-2 mediated neuroinflammation on the neurodegeneration and cognitive impairments in colchicine induced rat model of Alzheimer's 10.002. [58] G. Kaka, K. Yaghoobi, S. Davoodi, S.R. Hosseini, Assessment of the neuroprotective effects of Lavandula angustifolia extract on the contusive model of spinal cord injury in wistar rats, Front. Neurosci. 10 (2016) 1–9, https://doi.org/10.3389/fnins. 2016.00025.

[53] X. Kang, J. Qiu, Q. Li, K.A. Bell, Y. Du, D.W. Jung, J.Y. Lee, J. Hao, J. Jiang, Cyclooxygenase-2 contributes to oxidopamine-mediated neuronal inflammation and injury via the prostaglandin E2 receptor EP2 subtype, Sci. Rep. 7 (2017) 1–14, https://doi.org/10.1038/s41598-017-09528-z.

[54] J. Li, X. Zhang, H. Huang, Protective effect of linalool against lipopolysaccharide/d-galactosamine-induced liver injury in mice, Int. Immunopharmacol. 23 (2014) 523–529, https://doi.org/10.1016/j.intimp.2014.10.001.

[55] A.T. Peana, S. Marzocco, A. Popolo, A. Pinto, (-)-Linalool inhibits in vitro NO formation: probable involvement in the antinociceptive activity of this monoterpene compound, Life Sci. 78 (2006) 719–723, https://doi.org/10.1016/j.lfs.2005.05. 065.

[56] L.F.S. Brum, T. Emanuelli, D.O. Souza, E. Elisabetsky, Effects of linalool on glutamate release and uptake in mouse cortical synaptosomes, Neurochem. Res. 26 (2001) 191–194, https://doi.org/10.1023/A:1010904214482. 10.1016/0048-3575(84)90008-7.

[57] V.M. Linck, A.L. Da Silva, M. Figueiró, E.B. Caramão, P.R.H. Moreno, E. Elisabetsky, Effects of inhaled Linalool in anxiety, social interaction and aggressive behavior in mice, Phytomedicine 17 (2010) 679–683, https://doi.org/10.1016/j.phymed

CHAPTER 5

Inverse Phosphatidylcholine/ Phosphatidylinositol Levels as Peripheral Biomarkers and Phosphatidylcholine/ Lysophosphatidylethanolamine-Phosphatidylserine as Hippocampal Indicator of Postischemic Cognitive Impairment in Rats.

Angélica María Sabogal-Guáqueta[1], Javier Gustavo Villamil-Ortiz[1], Julian D. Arias-Londoño[2], Gloria Patricia Cardona-Gómez[1]

[1]Neuroscience Group of Antioquia, Cellular and Molecular Neurobiology Area - School of Medicine, Sede de Investigación Universitaria (SIU), University of Antioquia, Medellín, Colombia
[2]Department of Systems Engineering, University of Antioquia, Medellín, Colombia

Frontiers in Neuroscience (2018) 1-16. doi: 10.3389/fnins.2018.00989

Abstract

Vascular dementia is a transversal phenomenon in different kinds of neurodegenerative diseases involving acute and chronic brain alterations. Specifically, the role of phospholipids in the pathogenesis of dementia remains unknown. In the present study, we explored phospholipid profiles a month postischemia in cognitively impaired rats. The two-vessel occlusion (2-VO) model was used to generate brain parenchyma ischemia in adult male rats confirmed by alterations in myelin, endothelium, astrocytes and inflammation mediator. A lipidomic analysis was performed via mass spectrometry in the hippocampus and serum a month postischemia. We found decreases in phospholipids (PLs) associated with neurotransmission, such as phosphatidylcholine (PC 32:0, PC 34:2, PC 36:3, PC 36:4, and PC 42:1), and increases in PLs implied in membrane structure and signaling, such as lysophosphatidylethanolamine (LPE 18:1, 20:3, and 22:6) and phosphatidylserine (PS 38:4, 36:2, and 40:4), in the hippocampus. Complementarily, PC (PC 34:2, PC 34:3, PC 38:5, and PC 36:5) and ether-PC (ePC 34:1, 34:2, 36:2, 38:2, and 38:3) decreased, while Lyso-PC (LPC 18:0, 18:1, 20:4, 20:5, and LPC 22:6) and phosphatidylinositol (PI 36:2, 38:4, 38:5, and 40:5), as neurovascular state sensors, increased in the serum. Taken together, these data suggest inverse PC/LPC-PI levels as peripheral biomarkers and inverse PC/LPE-PS as a central indicator of postischemic cognitive impairment in rats.

Keywords: Global ischemia, cognitive impairment, phospholipid profile, biomarkers, serum, hippocampu

Inverse Phosphatidylcholine/Phosphatidylinositol Levels as Peripheral Biomarkers and Phosphatidylcholine/Lysophosphatidylethanolamine-Phosphatidylserine as Hippocampal Indicator of Postischemic Cognitive Impairment in Rats

Introduction

Cognitive impairment and dementia are common phenomena induced by acute and chronic brain injury, including Alzheimer's disease, stroke, and traumatic brain injury (TBI) (Nucera and Hachinski, 2018). Additionally, these conditions are predisposed to or worsened by poor lifestyles, including obesity, metabolic disorders, sedentary habits, smoking, and cardiocerebrovascular diseases, among others (Moskowitz et al., 2010). Long-term deprivation of oxygen and glucose via hypoperfusion or vasoconstriction of small vessels generates neurovascular unit injury, which is associated with vascular dementia after focal or global cerebral ischemic injury in response to cardiac arrest, coronary artery bypass surgery, cardiorespiratory failure, and other conditions due to drastic reductions in blood flow to the brain (Llinas et al., 2000; Vijayan et al., 2017).

In particular, the incidence of cerebrovascular disease, the third leading cause of death and the first leading cause of physical and mental disability worldwide, is increasing in developing countries (Cardona-Gómez and Lopera, 2016). Particularly, brain ischemia is caused by the occlusion of blood vessels, depriving of oxygen and glucose, resulting in an energy failure that alters mitochondrial ATP synthesis and upregulates the production of oxidative stress, free radicals and lipid peroxidation. Also,, the activation of dopamine and glutamate, as excitatory neurotransmitters, induces intracellular calcium overload, metabolic dysfunction and acidosis (Moskowitz et al., 2010). The increase in intracellular calcium causes activation of enzymes involved in lipid metabolism, such as "sphingomyelinases and phospholipases A2, C, and D, that, in turn, promote the release of second messengers, such as diacylglycerol (DAG), phosphatidic acid (PA), and arachidonic acid (AA), involved in inflammation, excitotoxicity and other cell death pathways" (Phillis and O'Regan, 2004; Tian et al., 2009). However, biomarkers supporting the clinical diagnosis of stroke are in development, and the development of biomarkers used to diagnosis the risk of dementia after stroke and its prevention is a true challenge.

In regard to lipids, these compounds are diverse, complex, and their functions depend on cellular distribution. Lipids are crucial in the homeostasis of cell membrane structure and act as signaling molecules and modulators in the central nervous system (CNS) (Martinez-Gardeazabal et al., 2017). Interestingly membrane lipids can be damaged by lipolysis under ischemia and by peroxidation of polyunsaturated fatty acids (PUFAs) during reperfusion (Schaller and Graf, 2004). Particularly, phospholipids, which are known for their high concentrations in the brain, play an important role both in normal neuronal activity and in pathological processes, even in those associated with memory impairment (Miyawaki et al., 2016). Therefore, we focused on elucidating the changes in lipid profiles of the hippocampus and serum of rats with cognitive impairment induced by global ischemia, as a continue of our previous studies (Marosi et al., 2006).

Materials And Methods

Animal Procedures

"All of the animal procedures were performed in accordance with the ARRIVE guidelines, the Guide for the Care and Use of Laboratory Animals, 8th edition published by the National Institutes of Health (NIH) and the Colombian standards (law 84/1989 and resolution 8430/1993). These procedures were approved by the Ethics Committee for Animal Experimentation of the University of Antioquia, Medellin, Colombia.

Male Wistar albino rats from our in-house, pathogen-free colony in the vivarium at SIU (Sede de Investigación Universitaria), University of Antioquia, Medellin, Colombia were kept on a 12:12 h dark/light cycle and received food and water ad libitum. Special care was taken to minimize animal suffering and to reduce the number of animals used. Three-month-old rats weighing 400–450 g were used. The rats were randomly divided into two groups, namely, the control and ischemic groups. Nine (9) rats were used per experimental group for behavioral, lipidomic and immunostaining evaluation (Marosi et al., 2006).

Global Cerebral Ischemia (2 VO)

"The animals were anesthetized using ketamine (60 mg/kg) and xylazine (5 mg/kg) and received a 2–4% isofiurane and 96% oxygen mixture via an inhalation anesthesia machine. A variation of the global cerebral ischemic model was implemented, involving a 2-vessel occlusion (2-VO; (Marosi et al., 2006). The right common carotid artery (CCA) was permanently occluded using a 6.0-gauge nylon suture (Corpaul, Bogota, Colombia), and the left CCA was obstructed for 20 min using a vascular clip. After the 20 min, the vascular clip was removed to allow reperfusion. Sham control rats underwent the same procedure without the CCA occlusion. The animals were sacrificed a month postischemia for lipid analyses" (Becerra-calixto and Cardona-gómez, 2017).

Immunochemistry and Immunofluorescence

Twenty four hours after the last behavioral test, animals were perfused intracardially with paraformaldehyde at 4%. Brains were removed and postfixed 48 h. Coronal sections (50 mm) obtained from vibratome were permeabilized, with 0.3% Triton X-100 and blocked with 1% BSA in PBS, using a previously described protocols for immunohistochemistry and immunofluorescence (Marosi et al., 2006), for the following evaluated primary antibodies: anti-NeuN (mouse monoclonal, Millipore, 1:500) anti-GFAP (monoclonal anti-glial fibrillary acidic protein, Sigma, 1:1000), anti-PECAM-1 (rabbit Platelet Endothelial Cell Adhesion Molecule 1, Abcam, 1:500), anti-myelin PLP (rabbit, myelin Proteolipid protein, Abcam, 1:200), COX-2 (rabbit, Cyclooxygenase 2, Abcam, 1:500). For immunofluorescence tissue, we incubated for 90 min at room temperature with mouse Alexa Fluor 488- or Alexa Fluor 594- conjugated anti-rabbit secondary antibodies (1:1000; Molecular Probes, Eugene, OR, United States). The tissues incubated in the absence of primary antibody did not display immunoreactivity.

Morris Water Maze Test

"Nineteen days after ischemia, the animals were evaluated in the Morris water maze (MWM) teste during 10 days (n = 9 per group). The test was performed using a previously described method (Becerra-calixto and Cardona-gómez, 2017). Briefly, a black plastic tank was filled with water (22 ± 2°C), and visual cues around the room remained in a fixed position throughout the experiment. The hidden platform (12 cm in diameter) was submerged 3 cm below the water level during spatial learning and 1.5 cm above the surface of the water during the visible session. Six sessions or trials were performed. Each session consisted of four successive subtrials (30 s intertrial interval), and each subtrial began with the rat being placed pseudorandomly in one of four starting locations. Then, the animals were provided with a 48 h retention period, followed by a probe trial of spatial reference memory, in which the animals were placed in the tank without the platform for 90 s. The latency to reach the exact former location of the platform was recorded during the probe trial. Later, the platform was moved to a new location and the ability of the animals to learn the new location was measured by determining the latency in 4 sessions conducted in the same manner as the learning phase. The latency to reach the platform was evaluated using a visible platform to control for any differences in visual-motor abilities or motivation between the experimental groups; the animals that could not perform the task were excluded. An automated system (Viewpoint, Lyon, France) recorded the behavior of the animals" (Marosi et al., 2006).

Tissue Preparation and Lipid Extraction

A month postischemia, four (4) "animals were sacrificed via decapitation, and the hippocampus of each rat was dissected, immediately frozen in liquid nitrogen and stored at 80 ◦C until analysis. We performed the same procedure to obtain serum samples. The total lipids from the hippocampus and serum were extracted according to the FOLCH technique (Jordi Folch, 1957) using a mixture of 2 mL of chloroform ($CHCl_3$) and 1 mL of methanol (CH_3OH) in a 2:1 (v/v) ratio. Then, 0.005% butylated hydroxytoluene (BHT) was added, and this mixture was used to homogenize the hippocampus. Subsequently, 1 mL of 0.9% NaCl was added, and the mixture was centrifuged at 3000 rpm for 3 min. The organic layer (lower layer) was removed and transferred to a new glass tube. The solvents were evaporated, and the extract was lyophilized to remove excess humidity. Finally, the lipid composition was analyzed via mass spectrometry" (Villamil-Ortiz et al., 2016).

Mass Spectrometry

"An automated ESI-MS/MS approach was used, and data acquisition and analysis were carried out at the Kansas Lipidomics Research Center using an API 4000 TM and Q-TRAP (4000Qtrap) detection system as described previously (Villamil-Ortiz et al., 2016). This protocol allowed the detection and quantification of low concentrations of the polar lipid compounds. The molecules were determined by the mass/charge ratios, which were compared with the respective internal standard to determine which species of lipids were

present in the evaluated extract: 0.30 nmol 14:0 lysoPG, 0.30 nmol 18:0 lysoPG, 0.30 nmol di 14:0 PG, 0.30 nmol 14:0-lysoPE, 0.30 nmol 18:0-lysoPE, 0.60 nmol 13:0-lysoPC, 0.60 nmol 19:0-lysoPC, 0.60 nmol di 12:0-PC, 0.60 nmol di 24:1-PC, 0.30 nmol 14:0 lysoPA, 0.30 nmol 18:0 lysoPA, 0.30 nmol di14:0-PA, 0.30 nmol di20:0 (phytanoyl)-PA, 0.20 nmol di 14:0-PS, 0.20 nmol di Phy PS, 0.28 nmol 16:0-18:0 PI, and 0.10 nmol di 18:0-PI. The system detected a total of 12 different lipid species and their respective subspecies, which were identified by the number of carbons and the degree of unsaturation in the chain. The lipid concentration was normalized according to the molar concentration across all species for each sample, and the final data are presented as the mean mol%" (Villamil-Ortiz et al., 2016).

Profile of Other Lipid Fractions

Lipids were extracted from the hippocampus and serum using the Folch method. The solid-phase extraction (SPE) as described Bermudez-Cardona et al (Bermúdez-cardona and Velásquez-rodríguez, 2016), was used to separate cholesterol esters (CE), triglycerides (TG), and free fatty acids (FFA).

Statistical Analysis

The behavioral test comparisons between two groups were performed using Student's t-tests for parametric data or Mann–Whitney tests for nonparametric data. "The lipid levels of each sample were calculated by summing the total number of moles of all lipid species measured and then normalizing that total to mol%. Comparisons between groups were assessed either by one-way ANOVA, followed by the Tukey post hoc test, or the Kruskal–Wallis test, depending on the homoscedasticity and normality of the experimental data. Multivariate statistics were performed using principal component analysis (PCA) and a partial least squares discriminant analysis (PLS-DA)" (Barker and Rayens, 2003). "The PLS-DA was included because this analysis is particularly suitable for the analysis of datasets with a small number of samples and a large number of variables. The PLS-DA was carried out using the protocols described previously by our laboratory" (Villamil-Ortiz et al., 2016). The data are expressed as the mean the standard error of the mean. The statistical significance is indicated in the figures and tables.

Results

Morphological Changes, Cognitive Impairment, and Hippocampal Phospholipid Profile Changes a Month Postischemia

The abilities of spatial learning and memory were examined by using the Morris water maze test. We found that, even a month postischemia, ischemic rats significantly increased their latencies to locate the platform in the MWM from trial 3 to trial 6 (Figure 1A). In the memory test, it was found that rats with global ischemia showed significant deficits in locating the submerged escape platform compared with sham rats (Figure 1A). In addition, during the

Inverse Phosphatidylcholine/Phosphatidylinositol Levels as Peripheral Biomarkers and Phosphatidylcholine/Lysophosphatidylethanolamine-Phosphatidylserine as Hippocampal Indicator of Postischemic Cognitive Impairment in Rats

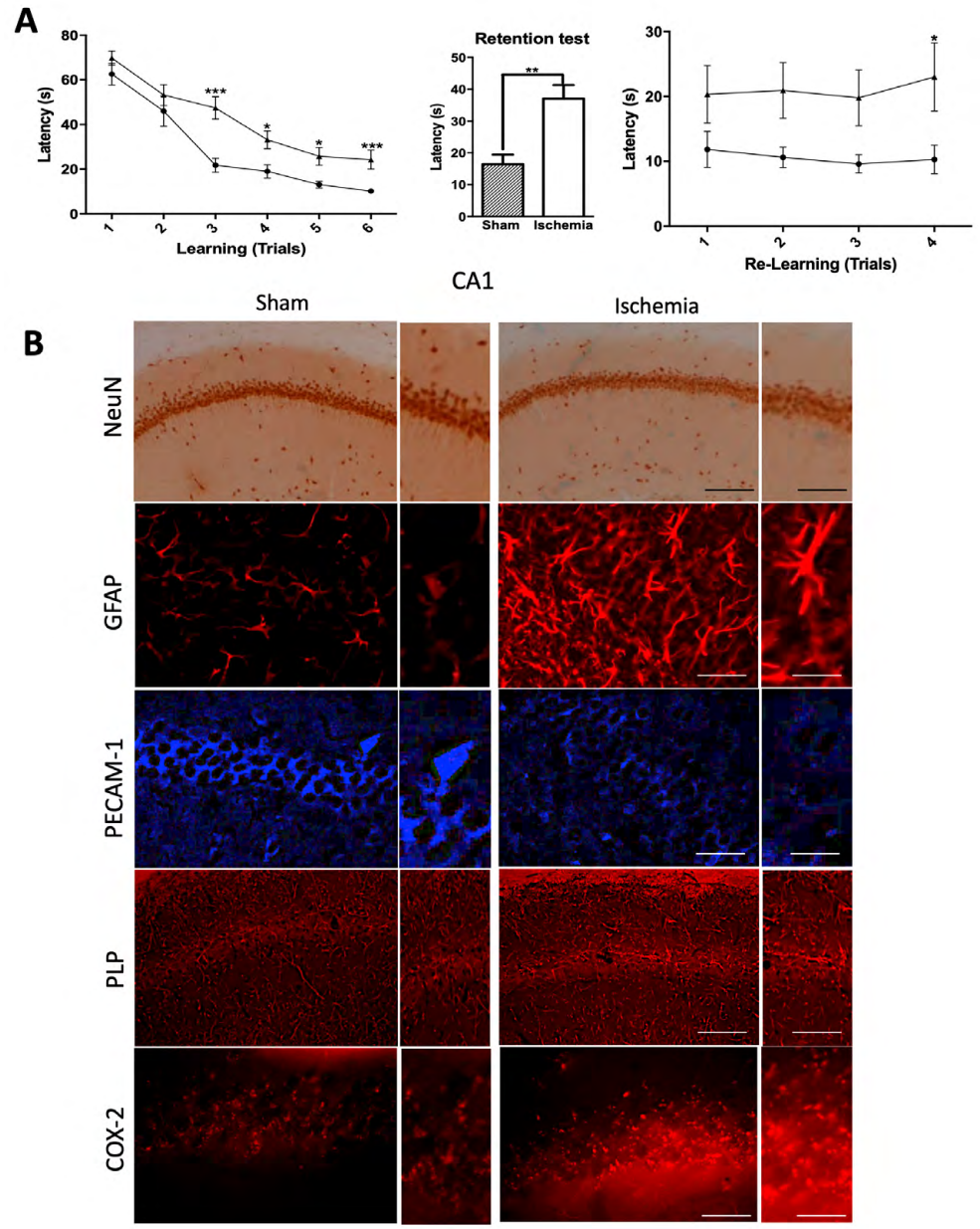


Figure 1. Morphological alterations and cognitive impairment in the rat hippocampus after transient global ischemia. (A) The learning and memory task performance was evaluated with the Morris water maze on day 19, starting with the learning test and the first position of the platform. The retention test was conducted after 48 h without the platform. The transference test included the second position of the platform. Data are expressed as group means ± SEM. ∗ $p < 0.05$, ∗∗ $p < 0.01$, and ∗∗∗ $p < 0.001$; n = 9 animals/group. (B) Immunostaining in pyramidal cell layer in the hippocampus with Neuronal nuclei (NeuN) immunohistochemistry; glial fibrillary acidic protein (GFAP), Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1), myelin Proteolipid protein (PLP), Cyclooxygenase 2 (COX-2) immunofluorescences. n = 5. NeuN and PLP, Magnification: 10×, scale bar: 100 µm; Insert: Magnification 40×; scale bar = 50 µm; GFAP, PECAM, and COX-2. Magnification 20×, scale bar: scale bar = 50 µm; Insert: 60×, scale bar: 15 µm.

relearning test, ischemic rats exhibited cognitive impairment a month postischemia, and the escape latencies were significantly lower compared with those of the sham group (Figure 1A). Which was supported by the morphological alterations of the hippocampus, although there was not a clear neuronal loss, we detected hypertrophic astrocytes with thickened processes and enlarged cell bodies (Figure 1B). PECAM-1, a cell adhesion marker, suggested disruption of the parenchima, PLP, a protein involved in the production of myelin, presented aggregation at the CA1, in addition to the increased presence of COX-2, inflammatory marker, suggesting the structural damage in the hippocampus at 1 month post global ischemia respect to healthy rats (Figure 1B).

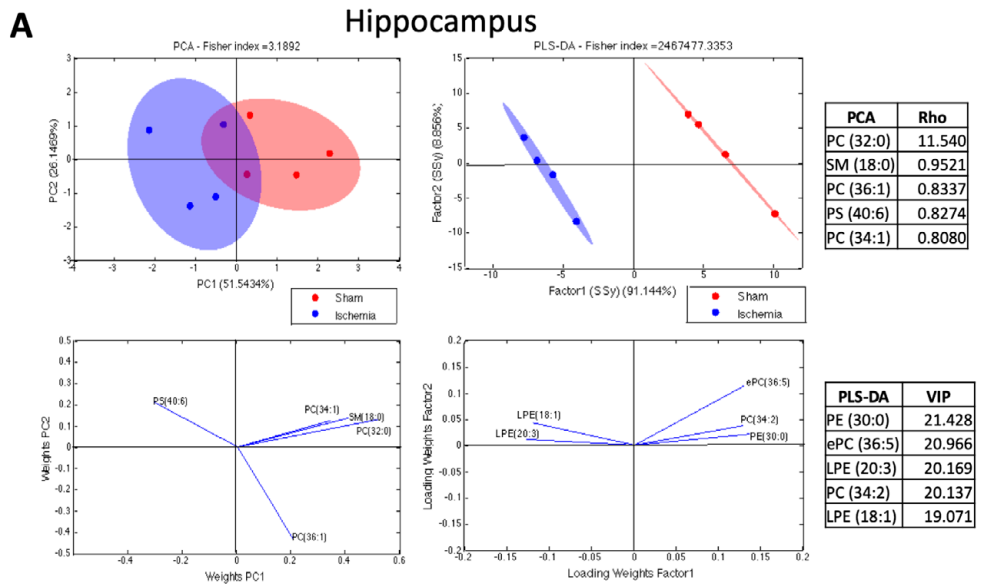
On the other hand, 311 species of phospholipids were evaluated via mass spectrometry in the hippocampus a month postglobal ischemia. At a glance, the analyses indicate that the changes seem to be related to the pathological condition. The lipid profiles of both groups show that they were primarily composed of high-abundance glycerophospholipids, such as PC (48.4 and 47%), PE (23.2 and 23.4%), PS (8.8 and 9.7%), and PI (3.8 and 3.9%); sphingolipids, such as SM-DSM (7.5 and 7.2%); low-abundance ether phospholipids, such as ePC (2.5 and 2.48%), ePE (2.48 and 2.5%), and ePS (0.02 and 0.03%); lysophospholipids, such as LPE (1.68 and 2.35%) and LPC (0.77 and 0.83%); and glycerophospholipids, such as PA (0.74 and 0.76%) and PG (0.11 and 0.12%) (Figures 2A,B).

The most relevant variables for these two components were related to reduced PC and increased PS subclasses (Figure 2B). The main Rho indices were PC 32:0, 34:1, and 36:1. PC 34:1 was composed of saturated palmitic acid (16:0) and oleic acid (18:1), while PC 32:0 was composed of two palmitic acids. In addition, PS 40:6, conformed by stearic acid (18:0) and docosahexaenoic acid (DHA) (22:6), was represented in the PCA. Complementarily, the PLS-DA showed ellipsoids that were completely different in terms of location; this could explain the reason that the control groups had a more diverse lipid composition compared with the ischemic group. The main changes were shown in LPE 18:1, 20:3, PE 30:0, PC 34:2, and ePC 36:5. Together these findings, based on the abundance by PCA and separability by PLS-DA, suggest decreased PC and increased PS and LPE composed of imbalanced saturated fatty acid (SFA), monounsaturated FA (MFA) and in polyunsaturated FA (PUFA) after ischemia.

Inverse PC and LPE/PS Levels in Postischemic Hippocampus of Rats

PCs are a class of 1,2-diacylglycerophospholipids that are essential components of cell membranes and have structural roles defined primarily by chain length (Billah and Anthes, 1990; Whiley et al., 2014). In previous studies using tMCAO (transient middle cerebral artery occlusion), it has been described that several PC species and sphingomyelin (SM) were significantly decreased after infarction in the cerebral cortex; in the same manner, LPCs were elevated in the tissue (Wang et al., 2010).

Inverse Phosphatidylcholine/Phosphatidylinositol Levels as Peripheral Biomarkers and Phosphatidylcholine/Lysophosphatidylethanolamine-Phosphatidylserine as Hippocampal Indicator of Postischemic Cognitive Impairment in Rats

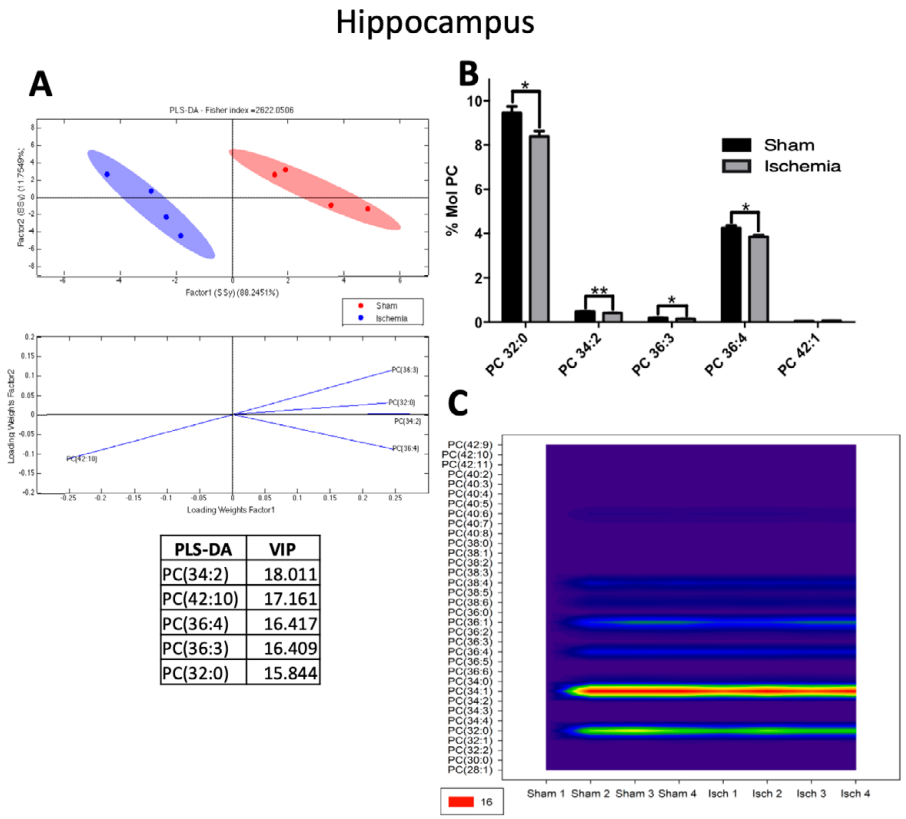


PCA	Rho
PC (32:0)	11.540
SM (18:0)	0.9521
PC (36:1)	0.8337
PS (40:6)	0.8274
PC (34:1)	0.8080

PLS-DA	VIP
PE (30:0)	21.428
ePC (36:5)	20.966
LPE (20:3)	20.169
PC (34:2)	20.137
LPE (18:1)	19.071

Figure 2. Phospholipid composition in the hippocampus after 1 month postischemia. (A) Multivariate analyses of the lipid profiles in the hippocampus. PCA and PLS-DA were performed to discriminate between the lipid classes. The left panel illustrates the factor loadings for PC1 and PC2 with the indices of variance explained for each component. The right panels show the factor score plots for the PLS-DA. (B) The lipid class profiles are expressed as % mol composition. All lipid species are represented as the means ± SEM. Data for ischemic group were significantly different from those of the control group ($^{*}p < 0.05$, $^{**}p < 0.01$; Student's t-test for parametric data or the Mann–Whitney test for nonparametric data, n = 4 per group).

We detected that global ischemia had a regulatory effect on the lipid profiles of PC and LPE from the hippocampus. The PLS-DA showed that the ischemic group had ellipsoids in different spaces, explained by the first component in 88% and the second one in 11% approximately (Figure 3A). In general, the PC levels decreased ($* p < 0.05$) in the ischemic groups compared with their counterpart in the sham control group (Figure 2B). Four PC subspecies showed significant reductions in global ischemia compared with those in the controls: 32:0 (16:0/16:0 - $p < 0.05$), 34:2 (16:1/18:1 ($p < 0.01$), 36:3 (18:1/18:2 - $p < 0.05$), and PC 36:4 (18:2/18:2) (Figures 3B,C). In general, considering that PC as the more abundant PL detected in the profile, those results were supported by the tendency and significant

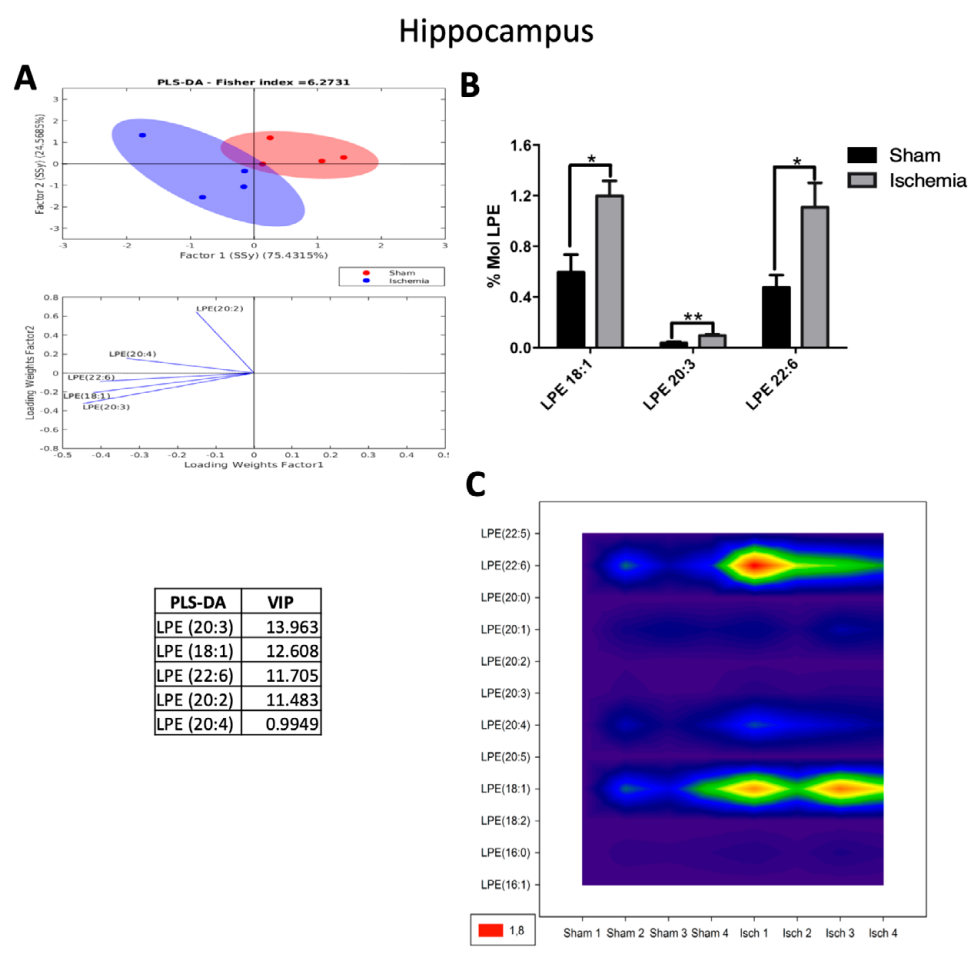


PLS-DA	VIP
PC(34:2)	18.011
PC(42:10)	17.161
PC(36:4)	16.417
PC(36:3)	16.409
PC(32:0)	15.844

Figure 3. Changes in phosphatidylcholine in the hippocampus after global ischemia. (A) Multivariate analyses: PLS-DA used to discriminate between the lipid classes in the hippocampus showing the factor score plots. (B) The PC profile is expressed as % mol composition. (C) Contour plots of the more influential subclasses of PC (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those of the control group and are represented as the means ± SEM (∗ $p < 0.05$, ∗∗ $p < 0.01$, and ∗∗∗ $p < 0.001$; Student's t-test for parametric data or the Mann–Whitney test for nonparametric data, n = 4 per group).

reduction of C.16:0 and C 18:0 at the total Free fatty acid (FFA) detected at the hippocampus and inversely C16:0 and C 18:0 were increased in the content of total triglycerides by the global ischemia. However, the CE did not change, lignoceric acid (24:0) FFA increased and a generalized reduction of oleic acid (18:1) was observed in the three analyzed fractions in the ischemic hippocampus (Supplementary Figures S1A–C).

On the other hand, LPE, a plasmalogen derived from phosphatidylethanolamine (PE), had significant higher levels ($p < 0.05$) in the ischemia group than in the sham group. The PLS-DA demonstrated that the ischemic group occupied a close area with respect to that of the sham group, having a small overlapping area, however, the separability was explained by the first component at a rate of 75.43% (Figure 4A). When the most differential and relevant phospholipidic species was LPE 18:1, 20:3, and 22:6 increased in the ischemic group (Figures 4A, B). These results were supported by the contour graphic, which showed increasing levels in these PL subclasses in the global ischemia group, mainly LPE 22:6 and 18:1 (Figure 4C).

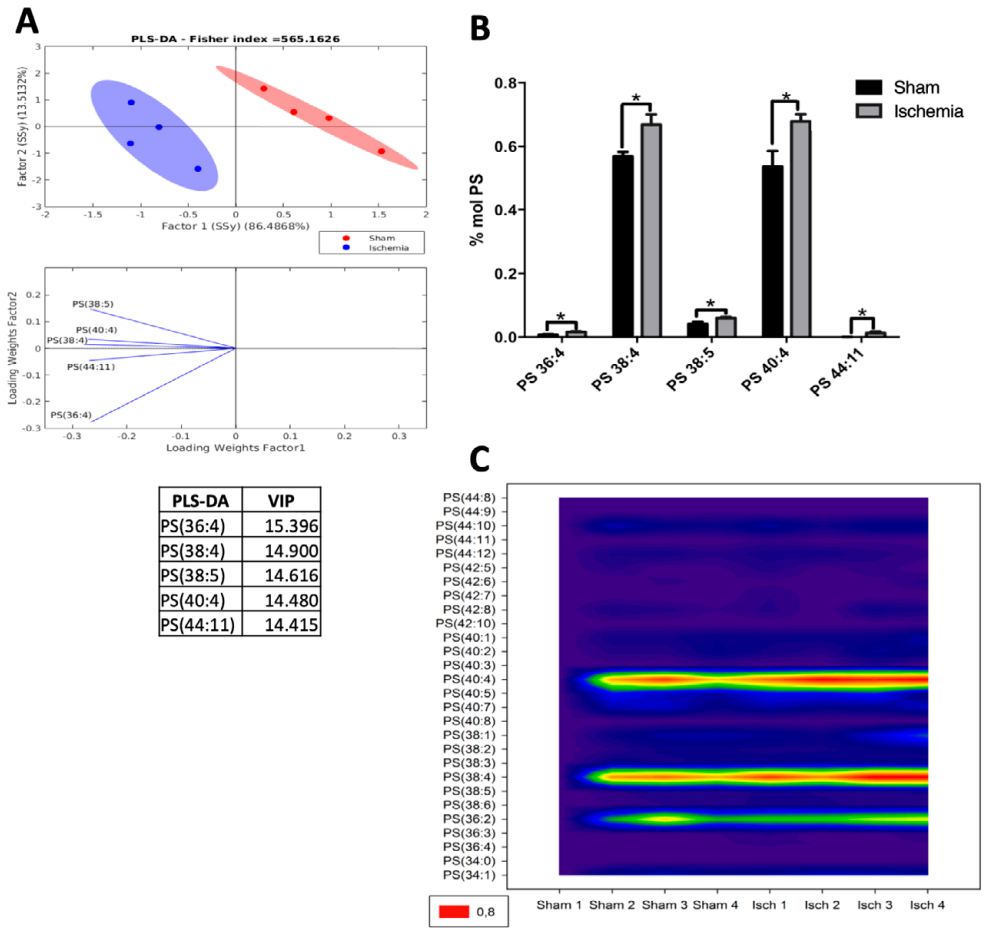


PLS-DA	VIP
LPE (20:3)	13.963
LPE (18:1)	12.608
LPE (22:6)	11.705
LPE (20:2)	11.483
LPE (20:4)	0.9949

Figure 4. Increase in LPE species in the hippocampus after global cerebral ischemia. (A) Multivariate analyses: PLS-DA used to discriminate between the lipid classes in the hippocampus showing the factor score plots. (B) The LPE profile is expressed as % mol composition. (C) Contour plots of the more influential subclasses of LPE (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different than those from the control group and are represented as the means ± SEM (∗ $p < 0.05$, ∗∗ $p < 0.01$, and ∗∗∗ $p < 0.001$; Student's t-test for parametric data or the Mann–Whitney test for nonparametric data, n = 4 per group).

Complementarily, PS modulates the binding properties of glutamate receptors involved in neurotransmission and long-term potentiation in the brain (Farooqui and Horrocks, 2007). The PLS-DA showed a different distribution of the ischemic group relative to that of the sham group in the PCA graph. These data showed that the main changes occurred in the subspecies of PC 36:4, 38:4, 40:4, 38:5, and 44:11, as was indicated by the VIP index (Figure 5A). Most of these subspecies are composed of arachidonic acid (20:4), a proinflammatory molecule. Given that their levels significantly increased in the ischemic group (∗ $p < 0.05$) (Figure 5B), this finding was in agreement with the high abundance shown in the counter plot graphic mainly by PS 40:4, 38:4, and 36:2 (Figure 5C).

Hippocampus



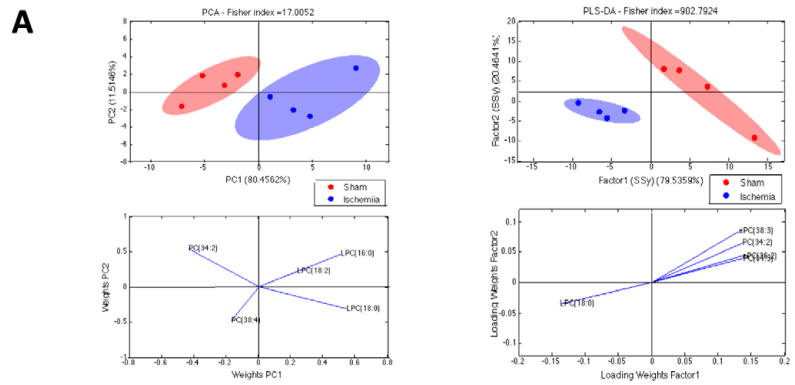
PLS-DA	VIP
PS(36:4)	15.396
PS(38:4)	14.900
PS(38:5)	14.616
PS(40:4)	14.480
PS(44:11)	14.415

Figure 5. PS species increased in the ischemic hippocampus. (A) Multivariate analyses: PLS-DA used to discriminate between the lipid classes in the hippocampus showing the factor score plots. (B) The PS profile is expressed as % mol composition. (C) Contour plots of the more influential subclasses of PS (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those from the control group and are represented as the means ± SEM (∗ $p < 0.05$ Student's t-test for parametric data or the Mann–Whitney test for nonparametric data, n = 4 per group).

Phospholipidic Profile Changes in the Serum a Month Postischemia in Rats

Evaluation of the lipid profiles of serum in ischemic rats with cognitive impairment a month postischemia could suggest potential biomarkers. The results of the PCA of the detected lipids indicated that nearly 92% of the total variance might be explained by the first two principal components (PC1 and PC2). The distribution pattern in the plane showed divergent distributions between the ischemic and sham groups in the lipid profiles of the serum (Figure 6A). Complementarily, the PLS-DA confirmed a displacement in the left quadrant of the ischemic group, while the sham group occupied a different region on the right side. PCA showed abundant and differential locations of LPC and PC between the control and ischemic

Serum



PCA	Rho
LPC(18:0)	172.941
LPC(16:0)	169.250
PC(34:2)	151.746
LPC(18:2)	79.475
PC(38:4)	70.916

PLS-DA	VIP
LPC(18:0)	18.344
PC(34:2)	18.248
PC(34:3)	18.053
ePC(38:3)	17.911
ePC(36:2)	17.902

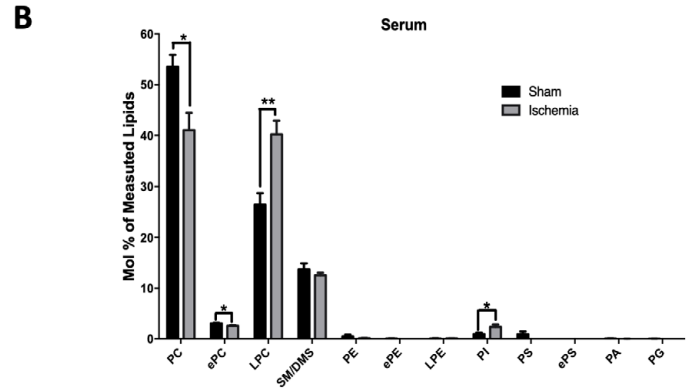
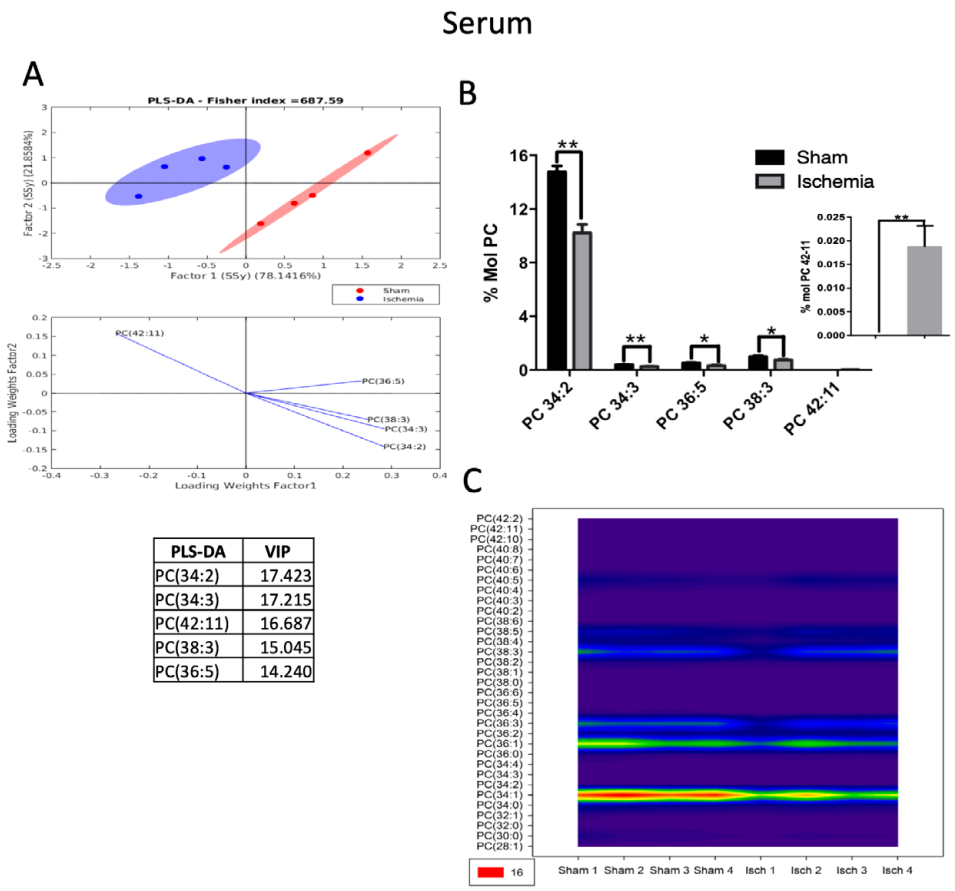


Figure 6. Altered phospholipid composition in the serum 1 month postischemia. (A) Multivariate analyses of the lipid profiles in the serum. PCA and PLS-DA were used to discriminate between the lipid classes. The left panel illustrates the factor loadings for PC1 and PC2 with the indices of variance explained for each component. The right panels show the factor score plots for the PLS-DA. (B) The lipid class profiles are expressed as % mol composition. All lipid species are represented as the means ± SEM. Data from the ischemic group were significantly different from those from the control group (∗ $p < 0.05$, ∗∗ $p < 0.01$, Student's t-test for parametric data or the Mann–Whitney test for nonparametric data, n = 4 per group).

groups, with the main divergent phospholipid species being LPC 18:0, 16:0 and PC 34:2 with a Rho of 172.9, 169.25, and 151.74, respectively. While PLS-DA also identified LPC 18:0 and PC 34:2 as the more discriminant species, with a VIP of 18.34 and 18.24, respectively, between others explained by the first component in approximately 80% (Figure 6A).

The lipid profiles of both groups showed that they were primarily composed of high-abundance glycerophospholipids, such as PC (53.3 and 47.9%) and LPC (26.43 and 23.55%); sphingolipids, such as SM-DSM (13.7 and 13.2%); low-abundance ether phospholipids, such



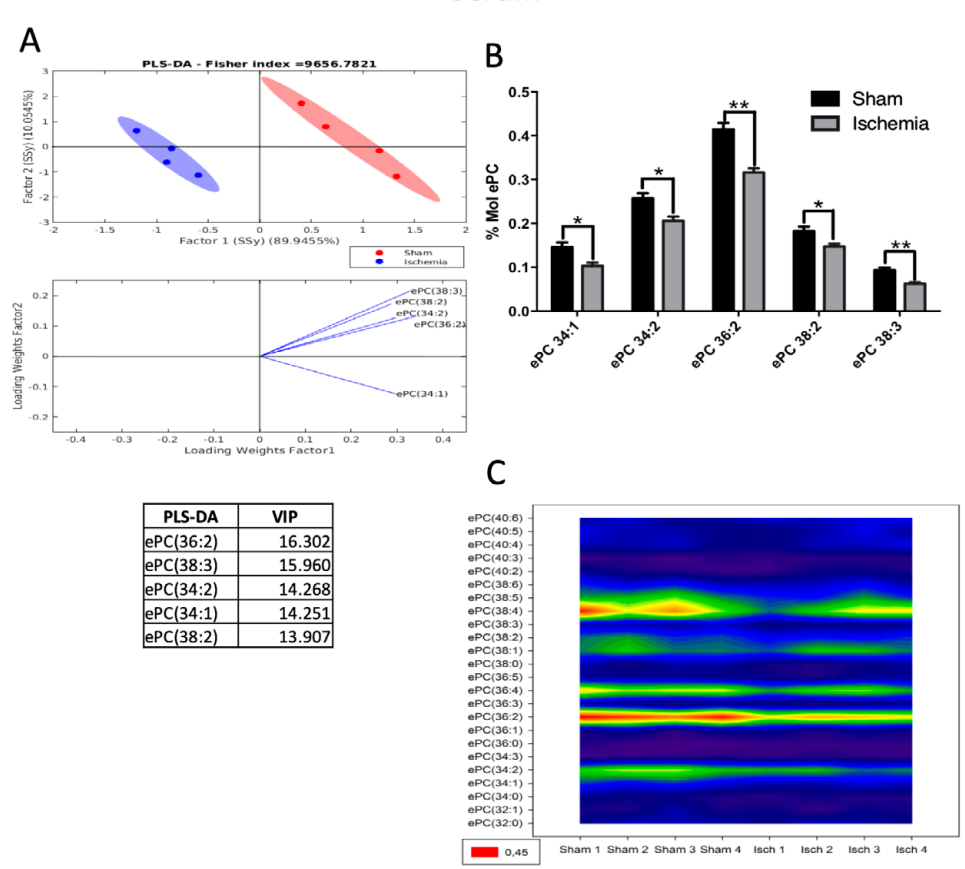
PLS-DA	VIP
PC(34:2)	17.423
PC(34:3)	17.215
PC(42:11)	16.687
PC(38:3)	15.045
PC(36:5)	14.240

Figure 7. The majority of the species of PC decreased in the serum of postischemic rats. (A) Multivariate analyses: PLS-DA analysis used to discriminate between the lipid classes in the serum showing the factor score plots. (B) The PC profile is expressed as % mol composition. (C) Contour plots of the more influential subclasses of PC (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those from the control groups and are represented as the means ± SEM (∗ $p < 0.05$, ∗∗ $p < 0.01$, and ∗∗∗ $p < 0.001$; Student's t-test for parametric data or the Mann–Whitney test for nonparametric data, n = 4 per group).

as ePC (3.07 and 2.85%), ePE (0.05 and 0.03%), and ePS (0.008 and 0.004%); PE (0.48 and 0.32%); PS (0.92 and 0.51%); PI (0.94 and 1.58%); LPE (0.068 and 0.074%); PA (0.05 and 0.04%) and PG (0.03 and 0.02%) (Figure 6B). In addition, these analyses showed that the main changes in serum were due to inverse levels of phosphatidylcholine and its plasmalogen, lysophosphatidylcholine, and the intermediate ePC species; together, a significant increase in PI subspecies occurred in the serum of the ischemic group.

Inverse PC and LPC-PI Serum Levels in Postischemic and Cognitively Impaired Rats

Our results indicated that lipid phosphatidylcholine molecule subspecies, such as PC 34:2, PC 34:3, PC 36:5, and PC 38:3, significantly decreased in the serum of the ischemic group, showing a notable separability (Figures 7A,B) and reduced abundance (Figure 7C) relative to

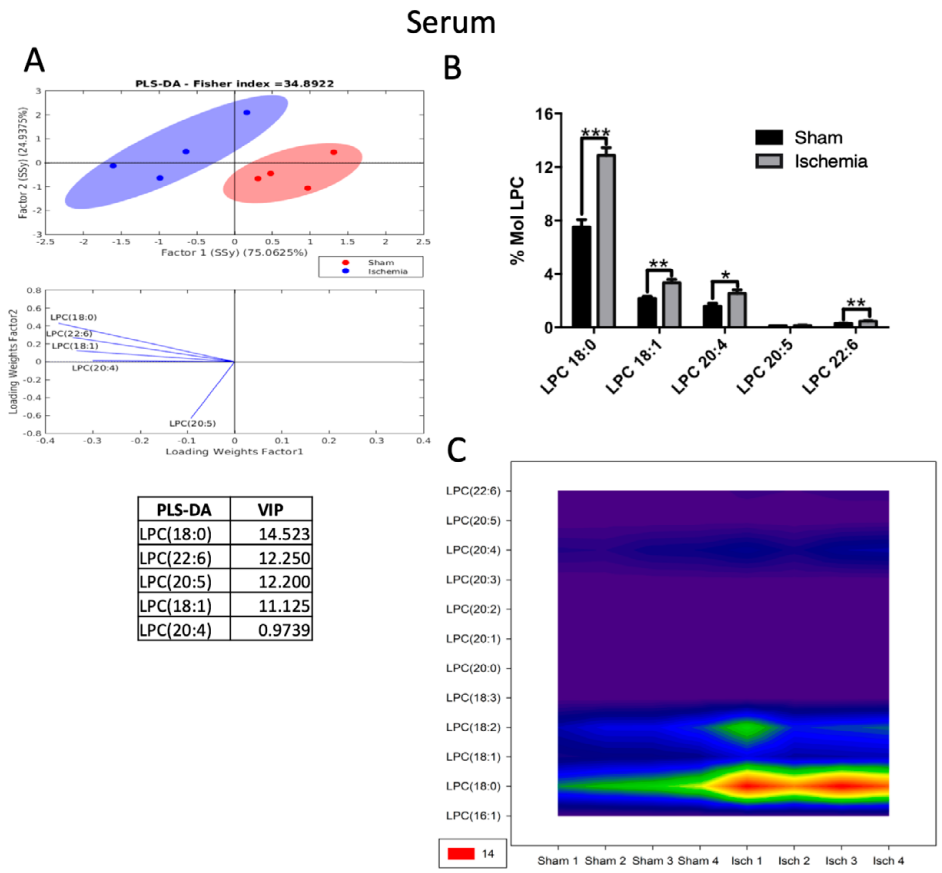


PLS-DA	VIP
ePC(36:2)	16.302
ePC(38:3)	15.960
ePC(34:2)	14.268
ePC(34:1)	14.251
ePC(38:2)	13.907

Figure 8. Changes in ePC in the serum of postischemic rats. (A) Multivariate analyses: PLS-DA analysis used to discriminate between the lipid classes in the serum showing the factor score plots. (B) The ePC profile is expressed as % mol composition. (C) Contour plots of the more influential subclasses of ePC (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those from the control group and are represented as the means ± SEM (∗ $p < 0.05$, ∗∗ $p < 0.01$, and ∗∗∗ $p < 0.001$; Student's t-test for parametric data or the Mann–Whitney test for nonparametric data, n = 4 per group).

those in the sham group (Figure 7). Similarly, we observed a reduction in ePC, as shown in Figure 8A, and the PLS-DA showed that the sham and ischemic rats had different patterns of distribution, and these changes were reflected by the following subspecies: ePC 36:2, ePC 38:3, ePC 34:2, ePC 34:1, and ePC 38:2 (Figure 8A), all of which were significantly reduced with respect to the levels observed in the control group (Figure 8B) and supported by the counter graph (Figure 8C). Those results could be supported by a general reduction of C 18 in the total FFA in serum (Supplementary Figures S1D–F), and no changes were detected in CE.

Inversely, LPC was shown to have increased in ischemic rats 1 month postischemia. The PLS-DA showed that ischemic and sham ellipsoids occupied different locations, supporting their different profiles (Figure 9A). The main subspecies that increased were LPC 18:0, LPC 22:6,

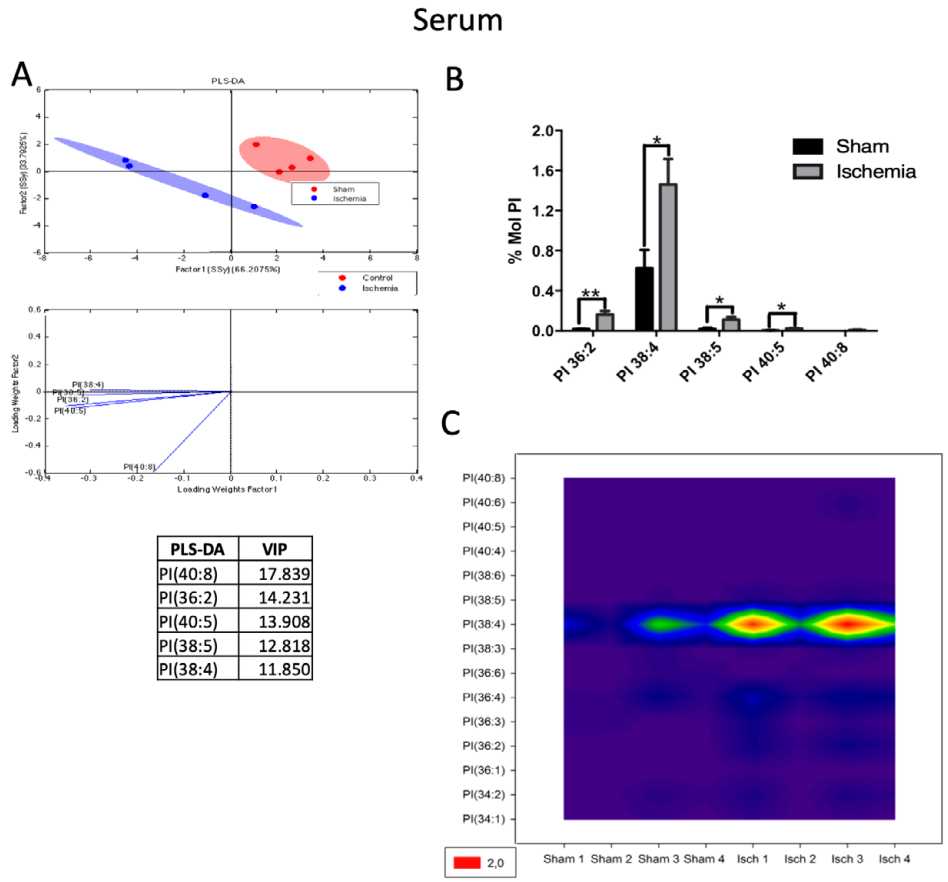


PLS-DA	VIP
LPC(18:0)	14.523
LPC(22:6)	12.250
LPC(20:5)	12.200
LPC(18:1)	11.125
LPC(20:4)	0.9739

Figure 9. Altered LPC in the serum after global ischemia. (A) Multivariate analyses: PLS-DA analysis used to discriminate between the lipid classes in the serum showing the factor score plots. (B) The LPC profile is expressed as % mol composition. (C) Contour plots of the more influential subclasses of LPC (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those from the control group and are represented as the means ± SEM (∗ $p < 0.05$, ∗∗ $p < 0.01$, and ∗∗∗ $p < 0.001$; Student's t-test for parametric data or the Mann–Whitney test for nonparametric data, n = 4 per group).

LPC 20:5, LPC 18:1, and LPC 20:4 (Figures 9A,B) in cases when LPC 18:0, which is a fatty acid involved in inflammatory processes in cerebrovascular disease, detected at a high level also by the counter graph (Figure 9C).

For its part, the following PI subspecies demonstrated increased levels in the ischemic group: PI 36: 2 (18: 1/18: 1); PI 38: 4 (18: 0/20: 4), and PI 38: 5 (18: 1 and 20: 4), with PI 38:4 being the most abundant according to the histogram and counter plot analyses (Figures 10A–C). Interestingly, PLs composed of fatty acids with long carbon chains, such as 18: 0 (stearic acid) 18: 1 (oleic acid) and 20: 4 (AA), are involved in proinflammatory processes and were detected in the serum of ischemic rats a month postinjury; maybe these results could be in relationship with the general increase of detected C 18:2 in TG and FFA fractions (Supplementary Figures S1E,F), future analysis should be done.



PLS-DA	VIP
PI(40:8)	17.839
PI(36:2)	14.231
PI(40:5)	13.908
PI(38:5)	12.818
PI(38:4)	11.850

Figure 10. **PI subspecies increased in the serum in postischemic rats.** (A) Multivariate analyses: PLS-DA analysis used to discriminate between the lipid classes in the serum showing the factor score plots. (B) The PI profile is expressed as % mol composition. (C) Contour plots of the more influential subclasses of PI (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those from the control group and are represented as the means ± SEM (∗ $p < 0.05$ Student's t-test for parametric data or the Mann–Whitney test for nonparametric data, n = 4 per group).

Discussion

Novelty, this study described hippocampal and peripheral phospholipid profile changes in long-term postischemia associated with cognitive impairment in rats. The main changes on PLs were associated to hippocampal dysfunction, represented by a downregulation of PC, as precursor of acetylcholine and inverse levels of LPE and PS associated with peroxisome damage, a proinflammatory environment and cell death in the hippocampal parenchyma after 1 month of anoxia, glutamate excitotoxicity and Brain Blood Barrier (BBB) disruption generated by global ischemia in rats (Becerra-calixto and Cardona-gómez, 2017). Also, these findings are supported by our recent study were proinflammatory phospholipid profile was associated to neurodegeneration and neurological dysfunction (Marosi et al., 2006).

Our data suggested that spatial learning and reference memory were significantly impaired in global ischemic rats, a finding that was in line with those of previous studies (Deng et al., 2017). These results demonstrated that the damage in ischemic rats is large, and the functional outcome can become worse over time. Recently, the high incidence of cognitive impairment after an ischemic stroke event has been described (Sun et al., 2014) and the comorbidity factors, such as atherosclerosis (Knopman et al., 2016). Additionally, plasma phospholipid changes have been suggested in cognitive impairment associated with brain stroke patients but have not been clearly identified by the variability in humans (Li et al., 2016). However, in subcortical ischemic vascular dementia and mixed dementia, an adaptative increase in polyunsaturated fatty acids and elevated membrane degradation (Lam et al., 2014) have been observed. With respect to mild cognitive impairment and its progression to dementia, this condition has been addressed in Alzheimer-type dementia, with lipidomic brain changes in serum PLs being observed, mainly via reductions in PC (Wood et al., 2016). The findings of these studies are in accordance with our current data from an experimental model of global ischemia, however, we showed a specific fatty acid composition imbalance constituted of 18:0, 18:1, 20:4, and 22:6 in the parenchyma and peripheral PLs profile changes in long-term postischemia associated with cognitive impairment. The fatty acid composition of imbalanced PLs in familiar and sporadic Alzheimer's disease in human brains has been commonly found, and in old triple transgenic AD mice with cognitive impairment, the imbalance has mainly been observed in PC and/or LPC, PE, and LPE (Villamil-Ortiz et al., 2016; Villamil-Ortiz et al., 2017).

Until now, few studies have focused on the impact of global ischemia on lipid signaling. Though some of these studies have investigated the first hours to 1 week of the acute phase of postischemia (Adibhatla and Hatcher, 2007), few have evaluated lipid profiles in the brain or serum, and none have focused on the potential relationship of these profiles with cognitive impairment. Therefore, our data are valuable for the detection of 12 lipid species, six of which had significant concentration changes in the hippocampus and serum, specifically PC, LPE, and PS in the hippocampus and PC, LPC, ePC and PI in the serum of ischemic and cognitively impaired rats during long-term postischemia, possibly suggesting differential lipid signatures under pathological conditions due to ischemia, which has been suggested by some related neurological studies (Lin and Perez-Pinzon, 2013; Shen et al., 2014; Hamazaki et al., 2016).

Glycerophospholipids are multifunctional molecules, are the major constituents of membranes and are responsible for the membrane bilayer, "mainly via choline or ethanolamine and to a lesser extent, inositol, serine or rarely, threonine. Further diversity is introduced by the components at the sn-1 and sn-2 positions, composing subclasses of diacyl and ether GP. Although plasmalogens represent up to 20% of the total phospholipid mass in humans, their physiological roles have been challenging to identify and are likely

Inverse Phosphatidylcholine/Phosphatidylinositol Levels as Peripheral Biomarkers and Phosphatidylcholine/Lysophosphatidylethanolamine-Phosphatidylserine as Hippocampal Indicator of Postischemic Cognitive Impairment in Rats

to vary in different tissues, metabolic processes and developmental stages" (Braverman and Moser, 2012). As plasmalogens serve as storage depots for second messengers and their precursors, membrane activity and ion channels, the study of plasmalogens may also provide insight into neural membrane pathology (Farooqui and Horrocks, 2007).

In particular, PC are composed of fatty acids with polyunsaturated chains, such as 34:2, 34:3, 36:4, 36:5, and 38:3, which are composed of linoleic acid (18:2), a precursor of the biosynthesis of AA (20:4) or AA composition per se (Choque et al., 2014), and may be supported by the increased LPC (18:0) catabolism to PC after ischemia. Additionally, elevated LPCs have been related to pathological lipid breakdown and the state of parenchymal inflammation after ischemia as an important source of reactive oxygen species (ROS) (Adibhatla and Hatcher, 2007; Wang et al., 2010), as well as being correlated with macrophage/microglia responses and neuronal death (Nielsen et al., 2016), spatial memory dysfunction (Köfeler et al., 2010) and its efflux and transport by ABCA7 in dementia by AD (Tomioka et al., 2017), also serving as a strategy in the forecast of ischemic stroke (Jickling and Montaner, 2015).

Furthermore, the lipid alterations found in our study suggested that phospholipid-modulating enzymes could be dysregulated in the brain, either due to increased or decreased phospholipase activity catalyzing the hydrolysis of LPC into PC. "The enzymes that catalyze the breakdown of PC to phosphatide (the phospholipase D or PLD enzymes) or to glycerophosphocholine and FFA (phospholipase A2 or PLA_2 enzymes) have been directly associated with cerebral ischemia. In addition, alterations in the reaction cascades of PLD enzymes, leading to aberrant phosphatidic acid (PA) signaling, have been linked to neurodegenerative processes, with the activation of PLA_2-family enzymes by β-amyloid peptide in neurons, in turn releasing secondary lipid messengers, such as AA. PLA_2s also play a role in the modification of physical properties, such as the fluidity of the cellular membrane" (Whiley et al., 2014). It is accepted that, during ischemia and reperfusion, free fatty acid concentrations increase, particularly those of polyunsaturated fatty acids released from membrane PLs through activation of PLA_2 (Hamazaki and Kim, 2013).

Another phospholipid that is highly involved is PS is the major acidic phospholipid in human membranes and one that constitutes 2–20% of the total phospholipid mass of adult human plasma and intracellular membranes (Van Meer et al., 2008). Hence, the presence of PS is essential for maintaining cell homeostasis, however, the fatty acid composition of PS is important, with studies of healthy human brains reporting that approximately 20–30% of the PS in human gray matter is composed of DHA (22:6), which is widely reported to be a pro-cell-survival fatty acid in the brain (Tanaka et al., 2012). Therefore, a reduction in the DHA content of PS is associated with the progression of mild cognitive impairment to dementia (Cunnane et al., 2012), possibly because the DHA in the PS conformation is essential for neuroprotection (Zhang et al., 2015). Additionally, PS synthesis may be inhibited

by metabotropic glutamate receptor agonists, indicating that metabotropic glutamate receptor stimulation decreases not only the incorporation of serine in PS but also modulates the generation of excitatory postsynaptic currents in rat cerebellar slices. In addition, in neural membrane, PS modulates long-lasting changes in learning and memory according to the membrane composition (Farooqui and Horrocks, 2007). Interestingly, in our study, the increased PS 1 month postischemia was mainly composed of polyunsaturated fatty acids, such as AA 20: 4 (36:4, 38:4, 40:4), possibly suggesting an imbalance between the DHA and AA concentrations in PS. In the context of cerebral ischemia, excess intracellular calcium (Ca_i^{2+}) activates various lipases, including (PLA_2) and PLC, which breakdown both intracellular and membrane phospholipids and release AA, thereby enhancing the proinflammatory response (Wang et al., 2007).

Interestingly, we also observed an increase in different species of LPE in the hippocampus of ischemic rats. LPE can be generated from PE via a phospholipase A-type reaction (Farooqui et al., 2000). Currently, the physiological significance of LPE in the brain after global ischemia is unknown. However, increased LPE has been demonstrated in major depressive disorder (MDD) and chronic stress (Liu et al., 2016; Oliveira et al., 2016). It has been reported that LPE has a direct relationship with calcium influx, which is closely related to cell death in neurodegenerative diseases and contributes to the cognitive impairment in transgenic mice with AD (Villamil-Ortiz et al., 2017).

For its part, phosphatidylinositol (PI) is characterized by the phosphorylation of the inositol head group of phosphoinositide, with a rapid and reversible phosphorylation rate, which critically participates in signal cascades and intracellular membrane trafficking (Hammond and Balla, 2015). PI is produced in the ER where its synthesizing enzymes, namely PI synthase (PIS) and CDP-DG synthase (CDS), are located (Kim et al., 2011). Based on the fact that PI is converted to PI3P in early endosomes and PI4P in the Golgi, plasma membrane (PM), and early and late endosomes, it is assumed that PI must be present in all of these membranes, as it has an important role in signaling pathways (Hammond and Balla, 2015). However, one past article mentioned that the four-vessel occlusion model-stimulated [3H] Inositol monophosphate formation via excitatory amino acids was greatly enhanced in hippocampal slices 24 h or 7 days after reperfusion (Seren et al., 1989). Additionally, PI has been recently reported to be a predictive marker of ischemic stroke (Tu et al., 2014).

In summary, our data showed that cognitive impairment in long-term postischemia is associated with a hippocampal phospholipid signature that indicates imbalance, proinflammatory environment, excitotoxicity and cell death, as evidenced in the peripheral PL profile related to cerebrovascular disruption and proinflammatory signaling and concomitantly supporting biomarkers of neurogenerative and cognitive impairment state and progression to long-term postischemia. Finally, this study provided a frame of reference

Inverse Phosphatidylcholine/Phosphatidylinositol Levels as Peripheral Biomarkers and Phosphatidylcholine/Lysophosphatidylethanolamine-Phosphatidylserine as Hippocampal Indicator of Postischemic Cognitive Impairment in Rats

for phospholipids that could be targeted for therapeutic exploration either through pharmacological intervention or enzymatic control, as phospholipases, for example, require further evaluation. Additionally, our findings are potentially useful for improving prediction and intervention after cerebral ischemia and form the basis for a future understanding of phospholipid dysfunction in neurological pathologies and targeting for prevention.

Acknowledgments

We thank the Cellular and Molecular Neurobiology Area of the Neuroscience Group of Antioquia for their scientific and technical support during the experiments.

Supplementary Material

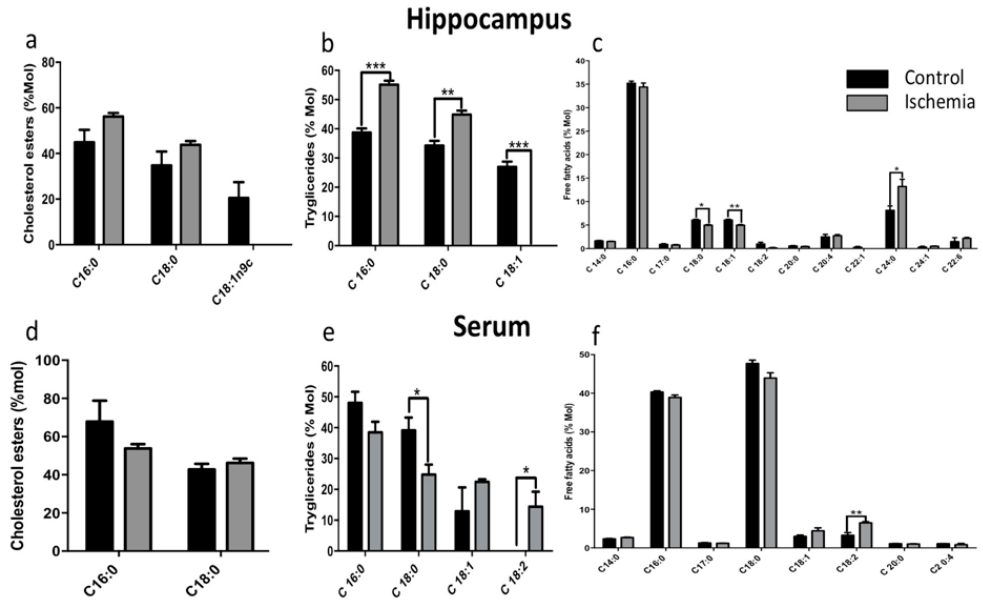


FIGURE S1. Total changes in lipid fractions from hippocampus and serum after global ischemia. Cholesterol esters (A,D) triglycerides (B,E), free fatty, acid (C,F) from hippocampus and serum, respectively, from ischemic and control rats are shown. Individual concentrations of lipids are expressed as molar percentage. Myristic acid (14:0), palmitic acid (16:0), margaric acid (17:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), arachidic acid (20:0), arachidonic acid (20:4), Erucic acid (22:1), Lignoceric acid (24:0), nervonic acid (24:1), docosahexaenoic, acid (22:6). Data represent means SEM of 4 mice per group. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$; for differences between sham and control groups.

Inverse Phosphatidylcholine/Phosphatidylinositol Levels as Peripheral Biomarkers and Phosphatidylcholine/Lysophosphatidylethanolamine-Phosphatidylserine as Hippocampal Indicator of Postischemic Cognitive Impairment in Rats

References

Adibhatla, R. M., and Hatcher, J. F. (2007). Role of lipids in brain injury and diseases. Future Lipidol. 2, 403–422. doi: 10.2217/17460875.2.4.403

Barker, M., and Rayens, W. (2003). Partial least squares for discrimination. J. Chemom. 17, 166–173. doi: 10.1002/cem.785

Becerra-calixto, A., and Cardona-gómez, G. P. (2017). Neuroprotection induced by transplanted CDK5 knockdown astrocytes in global cerebral ischemic rats. Mol. Neurobiol. 54, 6681–6696. doi: 10.1007/s12035-016-0162-2

Bermúdez-cardona, J., and Velásquez-rodríguez, C. (2016). Phospholipids, cholesterol esters and triglycerides metabolic syndrome. Nutrients 8, 1–13. doi: 10.3390/nu8020054

Billah, M. M., and Anthes, J. C. (1990). The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem. J. 269, 281–291. doi: 10.1042/ bj2690281

Braverman, N. E., and Moser, A. B. (2012). Functions of plasmalogen lipids in health and disease. Biochim. Biophys. Acta – Mol. Basis Dis. 1822, 1442–1452. doi: 10.1016/j.bbadis.2012.05.008

Cardona-Gómez, G. P., and Lopera, F. (2016). Dementia, preclinical studies in neurodegeneration and its potential for translational medicine in South America. Front. Aging Neurosci. 8:304. doi: 10.3389/fnagi.2016.00304

Choque, B., Catheline, D., Rioux, V., and Legrand, P. (2014). Linoleic acid: between doubts and certainties. Biochimie 96, 14–21. doi: 10.1016/j.biochi.2013.07.012

Cunnane, S. C., Schneider, J. A., Tangney, C., Tremblay-Mercier, J., Fortier, M., Bennett, D. A., et al. (2012). Plasma and brain fatty acid profiles in mild cognitive impairment and Alzheimer's disease. J. Alzheimers Dis. 29, 691–697. doi: 10.3233/JAD-2012-110629.Plasma

Deng, M., Xiao, H., Zhang, H., Peng, H., Yuan, H., Xu, Y., et al. (2017). Mesenchymal stem cell-derived extracellular vesicles ameliorates hippocampal synaptic impairment after transient global ischemia. Front. Cell. Neurosci. 11:205. doi: 10.3389/fncel.2017.00205

Farooqui, A. A., and Horrocks, L. A. (eds). (2007). "Phospholipases A2 in neurological disorders," in Glycerophospholipids in Brain (New York, NY: Springer), 394. doi: 10.1007/978-0-387-49931-4

Farooqui, A. A., Horrocks, L. A., and Farooqui, T. (2000). Glycerophospholipids in brain: their metabolism, incorporation into membranes, functions, and involvement in neurological disorders. Chem. Phys. Lipids 106, 1–29. doi: 10. 1016/S0009-3084(00)00128-6

Hamazaki, K., and Kim, H. Y. (2013). Differential modification of the phospholipid profile by transient ischemia in rat hippocampal CA1 and CA3 regions. Prostaglandins Leukot. Essent. Fat. Acids 88, 299–306. doi: 10.1016/j.plefa.2013. 01.003

Hamazaki, K., Maekawa, M., Toyota, T., Iwayama, Y., Dean, B., Hamazaki, T., et al. (2016). Fatty acid composition and fatty acid binding protein expression in the postmortem frontal cortex of patients with schizophrenia: a case-control study. Schizophr. Res. 171, 225–232. doi: 10.1016/j.schres.2016.01.014

Hammond, G. R., and Balla, T. (2015). Biochimica et biophysica acta polyphosphoinositide binding domains?: key to inositol lipid biology?. Biochim. Biophys. Acta 1851, 746–758. doi: 10.1016/j.bbalip.2015.02.013

Jickling, G. C., and Montaner, J. (2015). Lysophosphatidylcholine to stratify risk of ischemic stroke in TIA. Neurology 84, 17–18. doi: 10.1212/WNL.0000000000001100

Jordi Folch, S. S. (1957). A simple method for isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509. doi: 10.1016/j. ultrasmedbio.2011.03.005

Kim, Y. J., Guzman, M. L., and Balla, T. (2011). A highly dynamic ER-derived phosphatidylinositol synthesizing organelle suplies phosphoinositides to cellular membranes. Dev. Cell 21, 813–824. doi: 10.1016/j.devcel.2011.09. 005.A

Knopman, D. S., Gottesman, R. F., Sharrett, A. R., Wruck, L. M., Windham, B. G., Coker, L., et al. (2016). Mild cognitive impairment and dementia prevalence: the atherosclerosis risk in communities neurocognitive study, Alzheimer's Dement. 2, 1–11. doi: 10.1016/j.dadm.2015.12.002

Köfeler, H. C., Tiboldi, A., Hoeger, H., and Lubec, G. (2010). Hippocampal lipids linked to spatial memory in the C57bl/6j mouse. Neurochem. Int. 57, 935–939. doi: 10.1016/j.neuint.2010.09.013

Lam, S. M., Wang, Y., Duan, X., Wenk, M. R., Kalaria, R. N., Chen, C. P., et al. (2014). The brain lipidomes of subcortical ischemic vascular dementia and mixed dementia. Neurobiol. Aging. 35, 2369–2381. doi: 10.1016/j.neurobiolaging.2014.02.025

Li, D., Misialek, J. R., Boerwinkle, E., Gottesman, R. F., Sharrett, A. R., Mosley, T. H., et al. (2016). Plasma phospholipids and prevalence of mild cognitive impairment and/or dementia in the ARIC neurocognitive study (ARIC-NCS). Alzheimer's Dement. 3, 73–82. doi: 10.1016/j.dadm.2016.02.008

Lin, H. W., and Perez-Pinzon, M. (2013). The role of fatty acids in the regulation of cerebral vascular function and neuroprotection in Ischemia. CNS Neurol. Disord. 12, 316–324. doi: 10.2174/1871527311312030005

Liu, X., Li, J., Zheng, P., Zhao, X., Zhou, C., Hu, C., et al. (2016). Plasma lipidomics reveals potential lipid markers of major depressive disorder. Anal. Bioanal. Chem. 408, 6497–6507. doi: 10.1007/s00216-016-9768-5
Llinas, R., Barbut, D., and Caplan, L. R. (2000). Neurologic complications of cardiac surgery. Prog. Cardiovasc. Dis. 43, 101–112. doi: 10.1053/pcad.2000.9030

Marosi, M., Rákos, G., Robotka, H., Németh, H., Sas, K., Kis, Z., et al. (2006). Hippocampal (CA1) activities in Wistar rats from different vendors. Fundamental differences in acute ischemia. J. Neurosci. Methods 156, 231–235. doi: 10.1016/j.jneumeth.2006.03.010

Martinez-Gardeazabal, J., González de San Román, E., Moreno-Rodríguez, M., LLorente-Ovejero, A., Manuel, I., Rodríguez-Puertas, R., et al. (2017). Lipid mapping of the rat brain for models of disease. Biochim. Biophys. Acta 1859, 1548–1557. doi: 10.1016/j.bbamem.2017.02.011

Miyawaki, S., Imai, H., Hayasaka, T., Masaki, N., Ono, H., Ochi, T., et al. (2016). Imaging mass spectrometry detects dynamic changes of phosphatidylcholine in rat hippocampal CA1 after transient global ischemia. Neuroscience 322, 66–77. doi: 10.1016/j.neuroscience.2016.02.013

Moskowitz, M. A., Lo, E. H., and Iadecola, C. (2010). The science of stroke: mechanisms in search of treatments. Neuron 67, 181–198. doi: 10.1016/j. neuron.2010.07.002

Nielsen, M. M. B., Lambertsen, K. L., Clausen, B. H., Meyer, M., Bhandari, D. R., Larsen, S. T., et al. (2016). Mass spectrometry imaging of biomarker lipids for phagocytosis and signalling during focal cerebral ischaemia. Sci. Rep. 6:39571. doi: 10.1038/srep39571

Nucera, A., and Hachinski, V. (2018). Cerebrovascular and Alzheimer disease: fellow travelers or partners in crime? J. Neurochem. 144, 513–516. doi: 10.1111/jnc.14283

Oliveira, T. G., Chan, R. B., Bravo, F. V., Miranda, A., Silva, R., Zhou, B., et al. (2016). The impact of chronic stress on the rat brain lipidome, Mol. Psychiatry 21, 80–88. doi: 10.1038/mp.2015.14

Phillis, J. W., and O'Regan, M. H. (2004). A potentially critical role of phospholipases in central nervous system ischemic, traumatic, and neurodegenerative disorders. Brain Res. Rev. 44, 13–47. doi: 10.1016/j. brainresrev.2003.10.002

Schaller, B., and Graf, R. (2004). Cerebral ischemia and reperfusion: the pathophysiologic concept as a basis for clinical therapy. J. Cereb. Blood Flow Metab. 24, 351–371. doi: 10.1097/00004647-200404000-00001

Seren, M. S., Aldinio, C., Zanoni, R., Leon, A. F. (1989). Stimulation of inositol phospholipid hydrolysis by excitatory amino acids is enhanced in brain slices from vulnerable regions after transient global Ischemia. J. Neurochem. 53, 1700–1705. doi: 10.1111/j.1471-4159.1989.tb09233.x

Shen, H., Zhou, J., Shen, G., Yang, H., Lu, Z., and Wang, H. (2014). Correlation between serum levels of small, dense low-density lipoprotein cholesterol and carotid stenosis in cerebral infarction patients (65 years of age). Ann. Vasc. Surg. 28, 375–380. doi: 10.1016/j. avsg.2013.01.029

Sun, J. H., Tan, L., and Yu, J. T. (2014). Post-stroke cognitive impairment: epidemiology, mechanisms and management. Ann. Transl. Med. 2:80. doi: 10. 3978/j. issn.2305-5839.2014.08.05

Tanaka, K., Farooqui, A. A., Siddiqi, N. J., Alhomida, A. S., and Ong, W. Y. (2012). Effects of docosahexaenoic acid on neurotransmission. Biomol. Ther. 20, 152–157. doi: 10.4062/biomolther.2012.20.2.152

Tian, H., Qiu, T., Zhao, J., Li, L., and Guo, J. (2009). Sphingomyelinase-induced ceramide production stimulate calcium-independent JNK and PP2A activation following cerebral ischemia. Brain Inj. 23, 1073–1080. doi: 10.3109/ 02699050903379388

Tomioka, M., Toda, Y., Mañucat, N. B., Akatsu, H., Fukumoto, M., Kono, N., et al. (2017). Lysophosphatidylcholine export by human ABCA7. Biochim. Biophys. Acta 1862, 658–665. doi: 10.1016/j.bbalip.2017.03.012

Tu, W. J., Liu, X. Y., Dong, H., Yu, Y., Wang, Y., and Chen, H. (2014). Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1: a meaningful and independent marker to predict stroke in the Chinese population. J. Mol. Neurosci. 52, 507–514. doi: 10.1007/s12031-013-0206-2

Van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008). Membrane lipids: where they are and how they behave. Nat. Rev. Mol. Cell Biol. 9, 112–124. doi: 10.1038/nrm2330

Vijayan, M., Kumar, S., Bhatti, J. S., and Reddy, P. H., (2017). Molecular Links and Biomarkers of Stroke, Vascular Dementia, and Alzheimer's Disease. 1st Edn. Amsterdam: Elsevier Inc., doi: 10.1016/bs.pmbts.2016.12.014

Villamil-Ortiz, J. G., Barrera-Ocampo, A., Arias-Londoño, J. D., Villegas, A., Lopera, F., and Cardona-Gómez, G. P. (2017). Differential pattern of phospholipid profile in the temporal cortex from E280A-familiar and sporadic Alzheimer's disease brains. J. Alzheimer's Dis. 61, 209–219. doi: 10.3233/JAD-170554

Villamil-Ortiz, J. G., Barrera-Ocampo, A., Piedrahita, D., Velásquez-Rodríguez, C. M., Arias-Londoño, J. D., and Cardona-Gómez, G. P. (2016). BACE1 RNAi restores the composition of phosphatidylethanolamine-derivates related to memory improvement in aged 3xTg-AD mice. Front. Cell. Neurosci. 10:260. doi: 10.3389/fncel.2016.00260

Wang, H. Y., Liu, C. B., Wu, H. W., and Kuo, J. S. (2010). Direct profiling of phospholipids and lysophospholipids in rat brain sections after ischemic stroke. Rapid Commun. Mass Spectrom. 24, 2057–2064. doi: 10.1002/rcm

Wang, Q., Tang, X. N., and Yenari, M. A. (2007). The inflammatory response in stroke. J. Neuroimmunol. 184, 53–68. doi: 10.1016/j.jneuroim.2006. 11.014

Whiley, L., Sen, A., Heaton, J., Proitsi, P., García-Gómez, D., Leung, R., et al. (2014). Evidence of altered phosphatidylcholine metabolism in Alzheimer's disease. Neurobiol. Aging. 35, 271–278. doi: 10.1016/j.neurobiolaging.2013.08.001

Wood, P. L., Locke, V. A., Herling, P., Passaro, A., Vigna, G. B., Volpato, S., et al. (2016). Targeted lipidomics distinguishes patient subgroups in mild cognitive impairment (MCI) and late onset Alzheimer's disease (LOAD). BBA Clin. 5, 25–28. doi: 10.1016/j.bbacli.2015.11.004

Zhang, W., Liu, J., Hu, X., Li, P., Leak, R. K., Gao, Y., et al. (2015). n-3 polyunsaturated fatty acids reduce neonatal hypoxic/ischemic brain injury by promoting phosphatidylserine formation and Akt signaling. Stroke 46, 2943–2950. doi: 10.1161/STROKEAHA.115

CHAPTER 6

Transversal phenomenon in the brain parenchyma of dementias: Phospholipid profile analysis between CADASIL and sporadic Alzheimer disease

Sabogal-Guáqueta Angélica María[a], Arias-Londoño Julián David[b] , Gutierrez-Vargas Johanna[c] , Sepulveda-Falla D[d,e], Glatzel M[d], Villegas-Lanau Andrés[e] , Cardona-Gómez Gloria Patricia[a]*

[a]Cellular and Molecular Neurobiology Area, Group of Neuroscience, SIU, Faculty of Medicine. University of Antioquia UdeA, Calle 70 No. 52 – 21, Medellín, Colombia.
[b]Department of Systems Engineering, University of Antioquia UdeA, Calle 70 No. 52 – 21, Medellín, Colombia.
[c]Grupo del Adulto Mayor. Corporación Universitaria Remington. Medellín, Colombia
[d] Institute of Neuropathology, University Medical Center Hamburg-Eppendorf, Hamburg D-20246, Germany.
[e] Brain Biobank. Group of Neuroscience, SIU, Faculty of Medicine. University of Antioquia. Calle 70 No. 52 – 21, Medellín, Colombia.

Submitted

Abstract

Sporadic Alzheimer's disease (SAD) is the most common form of dementia, and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is the most frequent hereditary ischemic small vessel disease of the brain. Relevant biomarkers or specific metabolic signatures could provide powerful tools to manage these diseases. Therefore, the main goal of this study was to compare the postmortem temporal cortex gray matter, corpus callosum and cerebrospinal fluid (CSF) between a cognitively healthy group and CADASIL and SAD groups. We evaluated 352 individual lipids, belonging to 13 lipid subclasses, using mass spectrometry, and the lipid profiles were subjected to multivariate analysis to discriminate between the dementia groups (CADASIL and SAD) and healthy controls. Our data showed differential lipidic patterns between the pure vascular dementia groups, CADASIL and SAD, and the healthy controls. The main species of lipid profiles showing greater discrimination by partial least squares-discriminant analysis (PLS-DA) and a higher significance multivariate correlation (sMC) index were as follows: PS 44:7 (18:1/22:6)/LPE 18:2 in gray matter (GM); PE 32:2 (16:1/16:1)/PC 44:6 (18:0/22:6) in white matter (WM), and ePE 38:2 (18:0/20:2)/ePC 34:3 (14:0/20:3) in CSF. Those findings were supported by a high correlation profile of the phospholipids (PLs) between the pathologies in heat maps. Interestingly, common subphospholipidic species were obtained in both dementias when these brains were compared with healthy brains, such as PS 44:7/LPC 22:5 in GM, PE 32:2 in WM and PA 38:5/PC 42:7 in CFS. Nevertheless, the heat maps indicated other possibilities, including other sublipidic species with a lower weight index. Our findings suggest that PLs involved in neurotransmission alteration, connectivity impairment and inflammation response in GM, WM and CSF are a transversal phenomenon affecting dementias such as CADASIL and SAD independent of the etiopathogenesis, thus providing a possible common prodromal phospholipidic biomarker of dementia.

Key words: Phospholipids, biomarkers, gray matter, white matter, Cerebrospinal fluid, dementia.

Transversal phenomenon in the brain parenchyma of dementias: Phospholipid profile analysis between CADASIL and Sporadic Alzheimer disease

Introduction

SAD is the most common form of dementia, and CADASIL is the most frequent hereditary ischemic small vessel disease of the brain [7, 37]. Those diseases are forms of dementia and have similar characteristics, including damaged small blood vessels with protein deposits in different regions of the brain, protein deposits generated, in part, by gamma secretase activities, and affected people who develop symptoms at approximately 40–60 years of age with weakening dementia that progresses to complete incapacity at the end stage [50]. CADASIL is a hereditary small-artery disease caused by mutations in the NOTCH3 (Notch homolog 3) gene on chromosome 19. Pathogenetic mutations alter the number of cysteine residues in the extracellular domain of NOTCH3, which accumulates in the small arteries of affected individuals [12, 32, 72]. These vascular changes cause ischemic brain events that could lead to the main symptoms of CADASIL, characterized by migraine with aura, subcortical ischemic events, mood changes, apathy, cognitive impairment and epilepsy [59, 90].

SAD is manifested by progressive behavioral changes, the loss of recent memory, and decline in executive and cognitive functions [51]. The traditional AD pathology is associated with the accumulation of Aβ peptide [4, 86] and tau hyperphosphorylation, the loss of neurons and synaptic terminals, neuro-inflammation, reactive astrocytosis, microvascular disease, and increased cellular stress. However, the exact trigger and drivers for disease progression are not fully understood [74]. To understand the histopathological changes in neurodegenerative diseases, new lipidomic approaches have suggested interesting changes in brain phospholipid metabolism that could be connected with the pathogenesis of dementia type AD [44].

The brain is the most lipid-enriched organ in the human body (excluding adipose tissue). Brain cells avidly take essential fatty acids across the blood-brain barrier to satisfy their need to synthesize complex acyl-lipids [6, 67]. The plasma membranes of neural cells are highly enriched in cholesterol, sphingolipids and phospholipids (PLs). PLs in the brain are characterized by the highest level of structural complexity in their hydrophilic head groups. The notable heterogeneity in fatty acid composition reveals many different molecular species exerting structural and physiological roles in maintaining normal brain function [14,15]. A better understanding of the molecular changes in the lipidome of both SAD and CADASIL might help to clarify the commonalities between the dysregulation of lipid metabolism and pathogenesis, as well as the contribution of biomarkers in the pathogenesis in each of these diseases, or to identify a common phospholipid-related phenomenon in brain damage. Thus, in a cohort of patients with CADASIL and SAD,[29] we developed phospholipid profiles of the temporal cortex, white matter and CSF and compared them with those of healthy brain samples.

Materials and Methods

Human brain samples

Postmortem tissue and CSF were obtained from the University of Antioquia Brain´s BioBank. The consent forms were signed by the patients themselves or their next of kin, and this study was approved by the Bioethical Committee for Human Studies at the University of Antioquia. Five subjects with SAD, 5 subjects with CADASIL and 5 control subjects, categorized by age and cognitive function, were included. The characteristics of the individuals are shown in Table 1, and the postmortem index was the time lapse between the patient´s death and sampling. Histological/pathological classification was assessed in the Neuroscience group of Antioquia using the Braak stage to classify the degree of AD [9], NIA-RI criteria for the pathological diagnosis of AD´s "high likelihood" [29] and the Consortium to Establish a Registry for Alzheimer's Disease (CERAD), a neuropsychological battery developed to screen AD [24]. It comprises the following eight subtests: Mini-Mental State Examination (MMSE/30), Verbal Fluency (VF/24), Modified Boston Naming Test (BNT/15), Word List Learning (WLL/30), Word List Recall (WLR/10), Word Recognition Discriminability (WLRD/10), Constructional Praxis Copy (CP/11), and Constructional Praxis Recall (PR/11). The temporal cortex, white matter and CSF samples were flash frozen and stored at −80°C for biochemical analyses.

Sectioning and histology

Sections (50 µm) of formaldehyde-fixed embedded temporal cortices were cut by using a vibratome. The neuronal population morphology was evaluated using Nissl staining with toluidine blue (Sigma). Briefly, the sections were rinsed in distilled water and then were immersed in 1% toluidine blue. Next, the slides were dehydrated, immersed in xylene, and mounted in Depex. For immunostaining, the sections were rinsed in distilled water for 5 min and then were heated at 80°C for 20 min in 0.1 M citrate buffer at pH 9. After cooling, the sections were incubated with the primary antibodies against anti-MAP-2 (monoclonal mouse, 1:500; Sigma M9942), anti-Iba-1 (polyclonal rabbit, 1:500; Wako 019-19741), anti-GFAP (polyclonal rabbit, 1:500; Sigma G3893), claudin-5 (monoclonal mouse, 1:250; Ruo AC3C2) and Vimentin (polyclonal rabbit, 1:500; Abcam ab137321) overnight at 4°C. Thereafter, the sections were incubated with biotinylated secondary antibodies and with the avidin–biotin-peroxidase complex kit (Vector) with 3,3'-diaminobenzidine- 4HCl/H2O2 (DAB, Sigma, St. Louis, MO, USA) as a substrate. Negative controls were run identically except for the incubation with the primary antibody. The sections were then mounted and examined under a Nikon microscope (Eclipse E200, Kawasaki, Japan). The images were modified to a binary system and integrated densities (relative units), which were obtained for each image. The background was automatically subtracted in each image to quantify the relative intensity of immunostaining at 40×.

Transversal phenomenon in the brain parenchyma of dementias: Phospholipid profile analysis between CADASIL and Sporadic Alzheimer disease

Table 1. Demographic and pathologic data for control, CADASIL and SAD groups. SAD cases showed angiopathy only SAD* show vasculopathy. NA= not available; NAP= not applicable. B = Histological findings suggest the diagnosis of Alzheimer´s disease. C = Histologic findings indicate the diagnosis of Alzheimer´s disease. CADASIL-Notch3 mutation.

Diagnosis	Gender	Age of onset (years)	Age of death (years)	Postmortem time (hours)	Braak NF stage	NIA-RI	CERAD score
SAD	F	77	88	5.5	II	A2,B1,C2	B
SAD*	M	74	83	< 24	V	A3,B3,C3	C
SAD	F	76	81	2.8	IV	A2,B2, C3	C
SAD	F	75	90	4.5	II	A2,B1,C2	B
SAD	M	74	81	2.1	III	A2,B2,C3	C
CADASIL	M	61	65	3.9	NAP	NAP	NAP
CADASIL	F	52	58	3.5	NAP	NAP	NAP
CADASIL	F	45	47	2.1	NAP	NAP	NAP
CADASIL	F	NA	49	5.7	NAP	NAP	NAP
CADASIL	M	42	59	5.7	NAP	NAP	NAP
Control	M	NAP	60	< 24	NAP	NAP	NAP
Control	F	NAP	84	4.3	NAP	NAP	NAP
Control	F	NAP	67	3.8	NAP	NAP	NAP
Control	F	NAP	75	14.7	NAP	NAP	NAP
Control	M	NAP	69	6.8	NAP	NAP	NAP

Lipid analyses

Cerebral cortex (0.5 gr), white matter (0.5 gr) and CSF (0.5 ml) were processed individually according to the FOLCH technique for the extraction of lipids [26] using a mixture of 2 mL of chloroform ($CHCl_3$) and 1 mL of methanol (CH_3OH) in a 2:1 (v/v) ratio. Next, 0.005% butylated hydroxytoluene (BHT) was added, and this mixture was used to homogenize the samples. Consequently, 1 mL of 0.9% sodium chloride (NaCl) was added, and the mixture was centrifuged at 3,000 rpm for 3 min. The organic layer (lower layer) was removed and transferred to a new glass tube. The solvents were evaporated, and the extract was lyophilized to remove the excess humidity. Dry lipid in average was 11 mg, 13 mg and 2,5 mg for Cerebral cortex, white matter and CSF, respectively. Finally, the lipid composition was analyzed by mass spectrometry.

Mass spectrometry

An automated ESI-MS/MS method was used, and data acquisition and analysis was carried out at the Kansas Lipidomics Research Center using an API 4000™ and Q-TRAP (4000Qtrap) detection system as described previously [89]. This protocol allowed the detection and quantification of low concentrations of polar lipid compounds. The molecules were determined by the mass/charge ratios and then were compared with their respective internal standard to define which species of lipids were in the evaluated extract: 0.30 nmol of 14:0 lysoPG, 0.30 nmol of 18:0 lysoPG, 0.30 nmol of 14:0 PG, 0.30 nmol of 14:0-lysoPE, 0.30 nmol of 18:0-lysoPE, 0.60 nmol of 13:0-lysoPC, 0.60 nmol of 19:0-lysoPC, 0.60 nmol of 12:0-PC, 0.60 nmol of 24:1-PC, 0.30 nmol of 14:0 lysoPA, 0.30 nmol of 18:0 lysoPA, 0.30 nmol

of 14:0-PA, 0.30 nmol of 20:0 (phytanoyl)-PA, 0.20 nmol of 14:0-PS, 0.20 nmol of Phy PS, 0.28 nmol of 16:0-18:0 PI, and 0.10 nmol of 18:0-PI. The system identified 13 different lipid species and their respective subspecies, which were recognized by the number of carbons and degree of unsaturation of the chain. The lipid concentration was normalized by the molar concentration across all species for each sample, and the final data were presented as the mean % Mol.

Statistical analysis

The lipid levels for each sample were calculated by summing the total number of moles of all lipid species measured and then normalizing that total to % Mol. Comparisons among groups were assessed either by one-way ANOVA followed by Tukey's post hoc test or the Kruskal-Wallis test, depending on the normal distribution of the experimental data. Multivariate analysis was performed using partial least squares-discriminant analysis (PLS-DA) [(45)]. PLS-DA was included because it is particularly useful for the analysis of datasets with few samples and many variables. PLS-DA was carried out using the routines described by Ballabio & Todeschini [(5)] and was performed to identify differences among the study groups (SAD, CADASIL and healthy controls). An index representing the importance of the variables according to the first three components was estimated: the PLS-DA index used was called the variable importance in projection (VIP)[(53)], a weighted sum of the squares of the PLS weight that indicates the importance of each variable in the model and reflects the proportion of the explained variance weighted by the covariance between the predictor variables and dependent variable—i.e., the groups. For comparison, the recently proposed significance multivariate correlation (sMC) [(76)] index was also included in the analyses. sMC is similar to VIP but discards residual variance in the predictor variables that can be considered nonrelevant information to discriminate among the groups. The confidence ellipsoids per group and treatment were also included. Furthermore, PLS-DA was performed iteratively until the smallest subset of variables was identified to obtain separability among the confidence ellipsoids of the groups whenever such a separability was observed with the whole set of variables. To evaluate the similarities among the % Mol values of the lipid species, Pearson's linear correlation coefficient was also measured. The data from the univariate statistics were expressed as means ± standard error of the mean. The statistical significance is indicated in the figures.

Results

Dendrite retraction and microgliosis are common histopathologies in CADASIL and sporadic Alzheimer's disease

To assess the neural state of the analyzed postmortem tissue from the dementia and control groups, we used immunostaining for markers of neurons, astroglia, microglia and vessels. We observed no significant changes in the quantification of MAP-2 immunoreactivity (IR); however, we detected an aggregation pattern for MAP-2 in LI-III and LVI in the CADASIL

Transversal phenomenon in the brain parenchyma of dementias: Phospholipid profile analysis between CADASIL and Sporadic Alzheimer disease

group and dendrite loss with apparent disassembly of dendrites in the GM from LI to LVI in the SAD group compared with the control group. These findings were supported by the aggregation of neurons visualized by Nissl staining, exhibiting disorganization in the cortical layers and neuronal shrinkage compared with round and pale stained nuclei, which are typical of normal healthy cells (Fig. 1). Additionally, when we evaluated the microglia by Iba-1 IR, we detected significant hyperreactivity in the CADASIL and SAD groups in the layer I-III, as well as in layer IV-VI and WM in the CADASIL group, compared with those in the control group. (Fig. 1).

To evaluate astrocytes, we used the GFAP antibody and detected no changes in the immunoreactivity level, but we found notable differences in the staining pattern in all cortical layers and WM in the CADASIL group compared with those in the control group, as well as a drastic reduction of GFAP in the SAD group. Additionally, vimentin showed reduced immunostaining in the CADASIL group; however, vimentin was distributed in vessels and surrounding astrocytes in the CADASIL group and was significantly reduced in the GM and WM in the SAD group (Fig. 1). These observations were supported by the localization of claudin-5 to the length of the shrunken vessels (as rapes) in the CADASIL samples and almost any claudin-5 IR in SAD samples. These results suggest that increased BBB leakage in the dementia groups might be correlated with dendrite retraction and microgliosis as a product of the altered lipidomic profile that presents a common pattern between the dementia groups.

Phospholipid profile in the temporal cortex and discrimination between the dementia groups (CADASIL and SAD) and healthy brains

Three hundred fifty-two species of PLs were evaluated by mass spectrometry to understand the effects of CADASIL and SAD on the cerebral cortex phospholipid profile. The temporal cortex lipid profile was detected in 13 lipid species, where similar changes were obtained in the PL concentrations in the dementia groups (CADASIL and SAD) compared with those in the healthy group (C); major variability was found more often in SAD than in CADASIL profiles. The findings showed a significant increase in PC (C 34 vs CADASIL 50/SAD 38%Mol) compared with the reduction in lysophosphatidylcholine (LPC) (C 1,3 vs CADASIL 0,4 SAD 0,7%Mol). Furthermore, we observed increased concentrations of PE (C 7,6 vs CADASIL 16,1/SAD 13,7%Mol) and LPE (C 0,34 vs CADASIL 0,62/SAD 0,61%Mol) and decreased concentrations of PI (C 11,9 vs CADASIL 2,4/SAD 8,5%Mol), PS (34,5 vs CADASIL 10,1/SAD 18,2%Mol), PA (C 1,3 vs CADASIL 0,2/SAD 0,7%Mol) and PG (C 0,43 vs CADASIL 0,08/SAD 0,3%Mol) (Fig. 2A).

Partial least squares-discriminant analysis was used to discriminate PLs between the CADASIL/SAD patients and controls. Scores plots of the three factors were constructed to analyze the trends among the different groups. PLS-DA indicated that the three groups were located differently at the quadrant (Fig. 2B), showing different identities. The CADASIL and

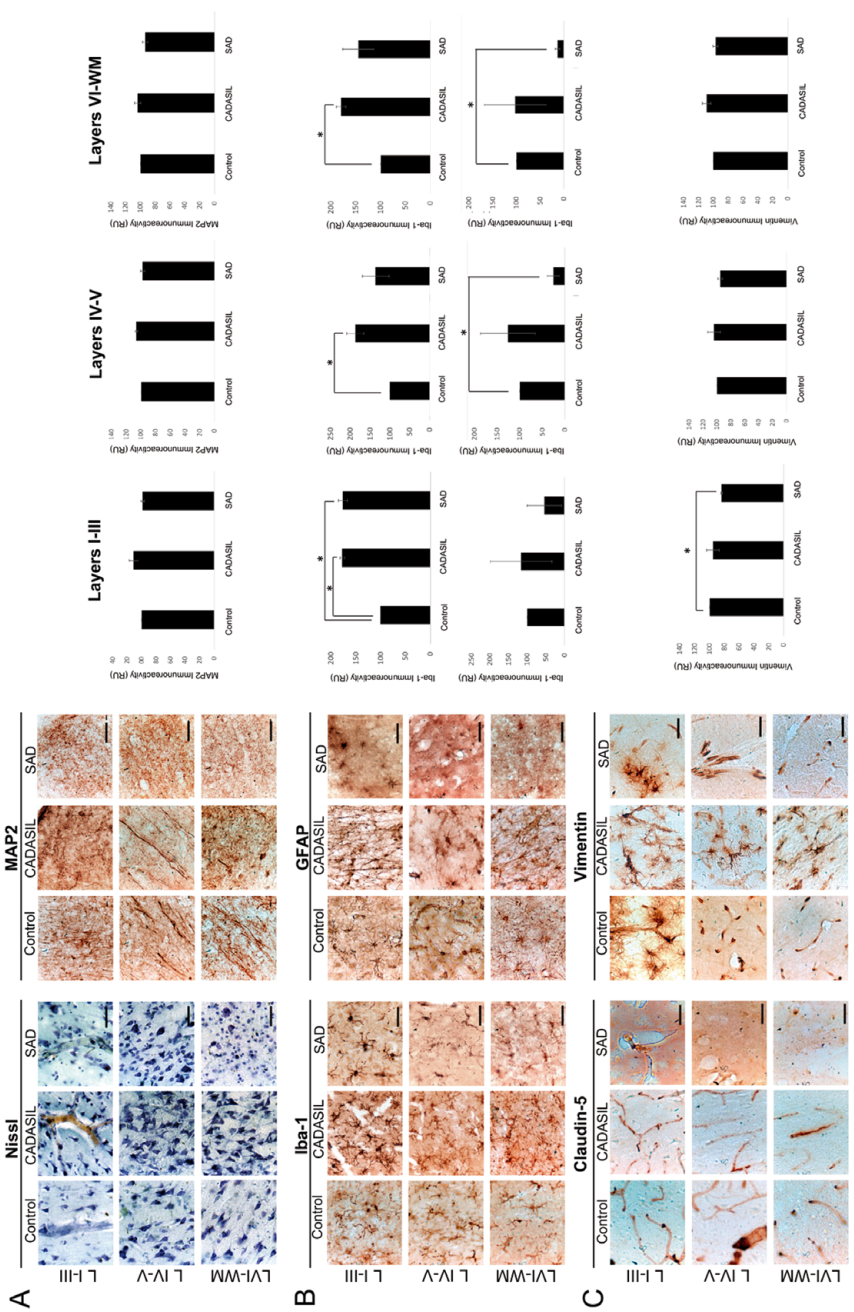
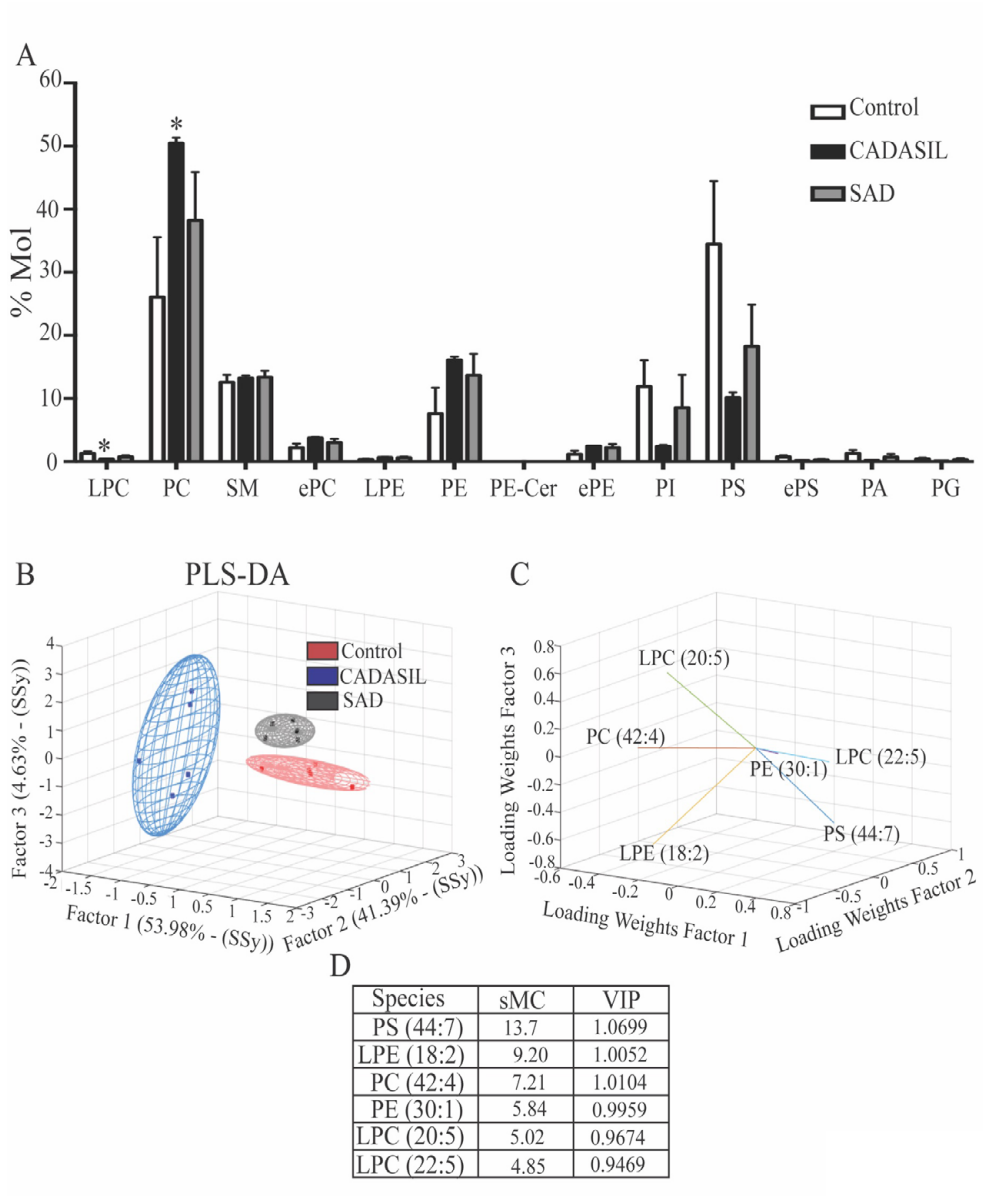


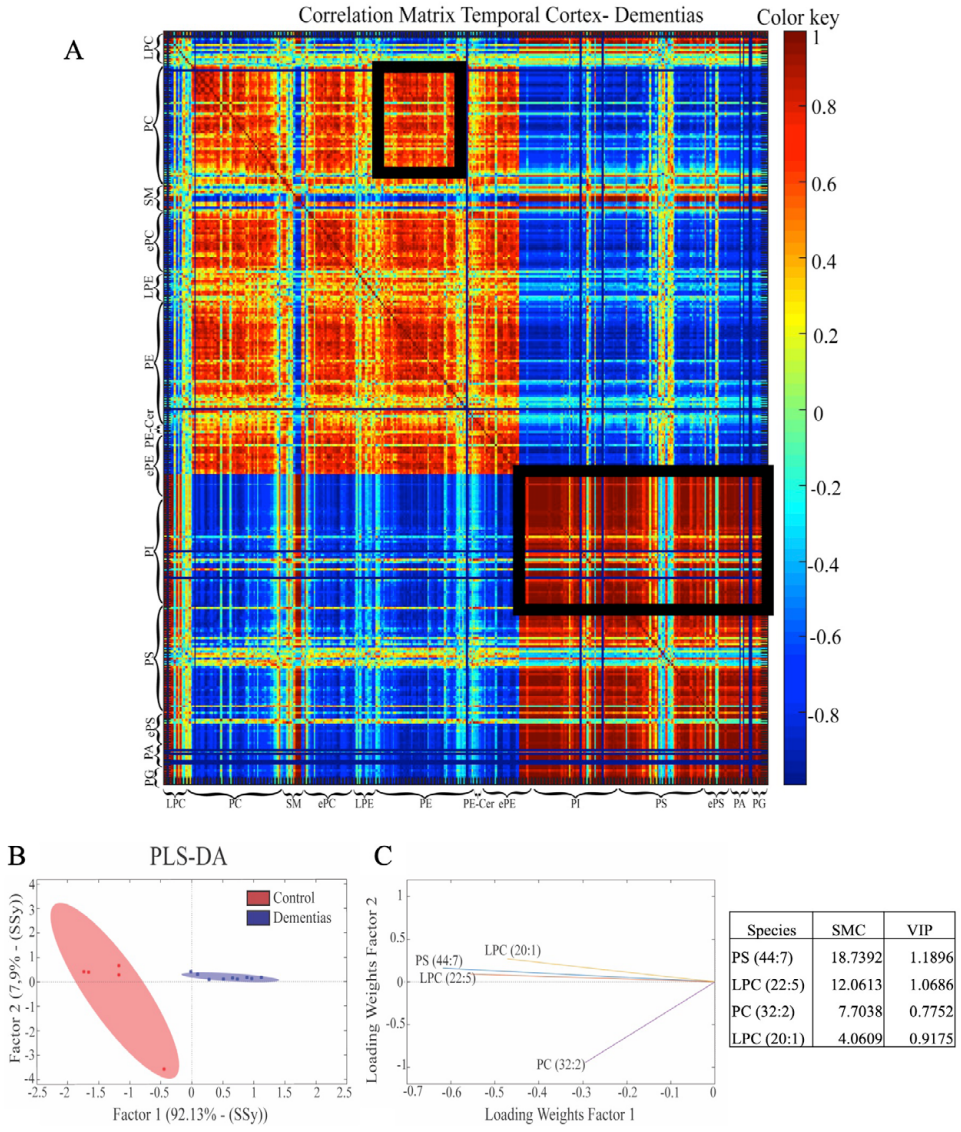
Figure 1. CADASIL and sporadic Alzheimer's present morphological alterations in dendrites, microglia and vascular endothelium. (A) Representative Nissl staining and MAP2, (B) Iba-1 and GFAP, (C) Claudin-5 and vimentin immunoreactivity levels in the cortex (layer I-VI) and white matter (WM) in control (C) CADASIL (CAD) and sporadic Alzheimer (SAD) patients. The micrographs were obtained at 40X. Scale bars= 25 µm (40X). RU: relative units. *Significant differences, $p \leq 0.05$; $n = 3$ /group.



D

Species	sMC	VIP
PS (44:7)	13.7	1.0699
LPE (18:2)	9.20	1.0052
PC (42:4)	7.21	1.0104
PE (30:1)	5.84	0.9959
LPC (20:5)	5.02	0.9674
LPC (22:5)	4.85	0.9469

Figure 2. Lipid composition of the temporal cortex from human CADASIL, SAD, and healthy subjects. A) The lipid class profiles are expressed as % Mol composition. All lipid species were measured (means), and the error bars represent the SEM. The data for CADASIL tissue were significantly different from the control subjects (∗p < 0.05, ANOVA followed by the Tukey post hoc test or Kruskal-Wallis test). B) PLS-DA analysis of the lipid profiles from the temporal cortex of CADASIL, SAD, and healthy subjects. The panel show the factor score plots for PLS-DA and C) show the projections of the data with the sMC and VIP metrics. D) Values from sMC and VIP in a table. LPC, Lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; ePC ether phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PE-Cer, Phosphatidylethanolamine Ceramide; ePE, ether phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; ePS, ether phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol. n = 5 per group.



Species	SMC	VIP
PS (44:7)	18.7392	1.1896
LPC (22:5)	12.0613	1.0686
PC (32:2)	7.7038	0.7752
LPC (20:1)	4.0609	0.9175

Figure 3. Correlation matrix in Dementias from Temporal Cortex. A) Heat map of the Pearson's linear correlation among the 352 measured metabolites. Metabolites are clustered using subspecies per families for CADASIL and SAD. B) PLS-DA analysis of the lipid profiles from dementia groups (CADASIL & SAD) with control group and C) show the projections of the data with the sMC and VIP metrics. n = 5 per group. LPC, Lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; ePC ether phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PE-Cer, Phosphatidylethanolamine Ceramide; ePE, ether phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; ePS, ether phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol. Black square: higher correlations between PI with PS, ePS, PA, PG

SAD groups showed a differential distribution pattern, indicating that the phospholipid (PL) profile was different for each type of dementia (Fig. 2B). Additionally, 53,9% of the total variance may be explained by factor 1, followed by the factor 2 with 41.29% and factor 3 with 4,63%. According to the sMC and VIP, the six most changing species were confirmed to be PS (44:7) (sMC: 13,17/VIP: 1,0699), LPE (18:2) (sMC: 9,20/VIP: 1,0052), PC (42:4) (sMC: 7,21 VIP: 1.0104), PE (30:1) (sMC: 5,84/VIP: 0.9959), LPC (20:5) (sMC: 5,02/VIP: 0,9674) and LPC (22:5) (sMC: 4,85/VIP: 0,9469), as shown in Fig. 2.C-D.

Similarities and differences of the lipidic subspecies in the temporal cerebral cortex among the CADASIL, SAD and healthy brain groups

Phosphatidylcholine (PC) is the most abundant phospholipid of all mammalian cell types and subcellular organelles and plays structural roles defined primarily by chain length [77]. To our best knowledge, this is the first description of a lipid profile for CADASIL. However, in previous studies in AD, several PC and LPC species were reported to be significantly decreased, increased or unchanged depending on the sample and technique used for the analysis [43, 46, 54].

A general overview revealed a significant increase in PC (p: 0,04), while LPC (p:0,0216) was reduced, in the CADASIL group compared with control in the temporal cortex. Additionally, PI and PS showed a clear decrease in the CADASIL group (Fig. 2A). The SAD group showed a similar tendency of changes compared with the CADASIL group. However, we observed no significative differences via ANOVA, probably because of the variability of the data.

Additionally, a heat map was computed to visualize the trends for the 352 lipid classes and correlation between dementia groups (Fig. 3A), showing that PC was correlated strongly with PE, and LPE, LPC with PS (highlighted square). Furthermore, we observed novelty correlations between PI and PS, ePS, PA, or PG (Fig. 3 A). When we performed PLS-DA considering both dementias in one group (SAD & CADASIL) compared with the controls, the figure showed ellipsoids, with a closer distribution between the dementias, and different quadrants, explained by the first component in 92,13% and second one in 7,9% (Fig. 3B). The four subspecies more discriminant of dementias compared with control were as follows: PS (44:7), LPC (22.5), PC (32:2) and LPC (20:1) (Fig. 3C). Concordantly, when we compared the CADASIL and control groups, the CADASIL group was fairly homogeneous, with the first component explaining the discrimination in 99,66% and second one in only 0,34%%: PC (42:4), PS (44.7), LPC (22:5) and PS (36:5) were different from those of the control (Fig. suppl 1A). Additionally, when we compared the SAD and control groups, the first component explained the discrimination in 98,59% and the second in 1,41%; some species similar to those in CADASIL were highlighted: PS (44:7), LPC (22:5), PS (44:4) and ePC (34:4) (Fig. suppl 1B). However, when we compared the CADASIL and SAD groups, we found that the first component explained the discrimination in 94.74% and the second in 5,26%. We detected

differences in LPE (18:2), PS (44:9), PA (34:3) and PS (42:8) in the CADASIL group compared with those in the SAD group (Fig. suppl 1C).

Phosphatidylserine disbalance comprising docosahexaenoic acid, erucic acid derivates and linoleic acid, and the similarities and differences between the CADASIL and SAD groups in the temporal cerebral cortex

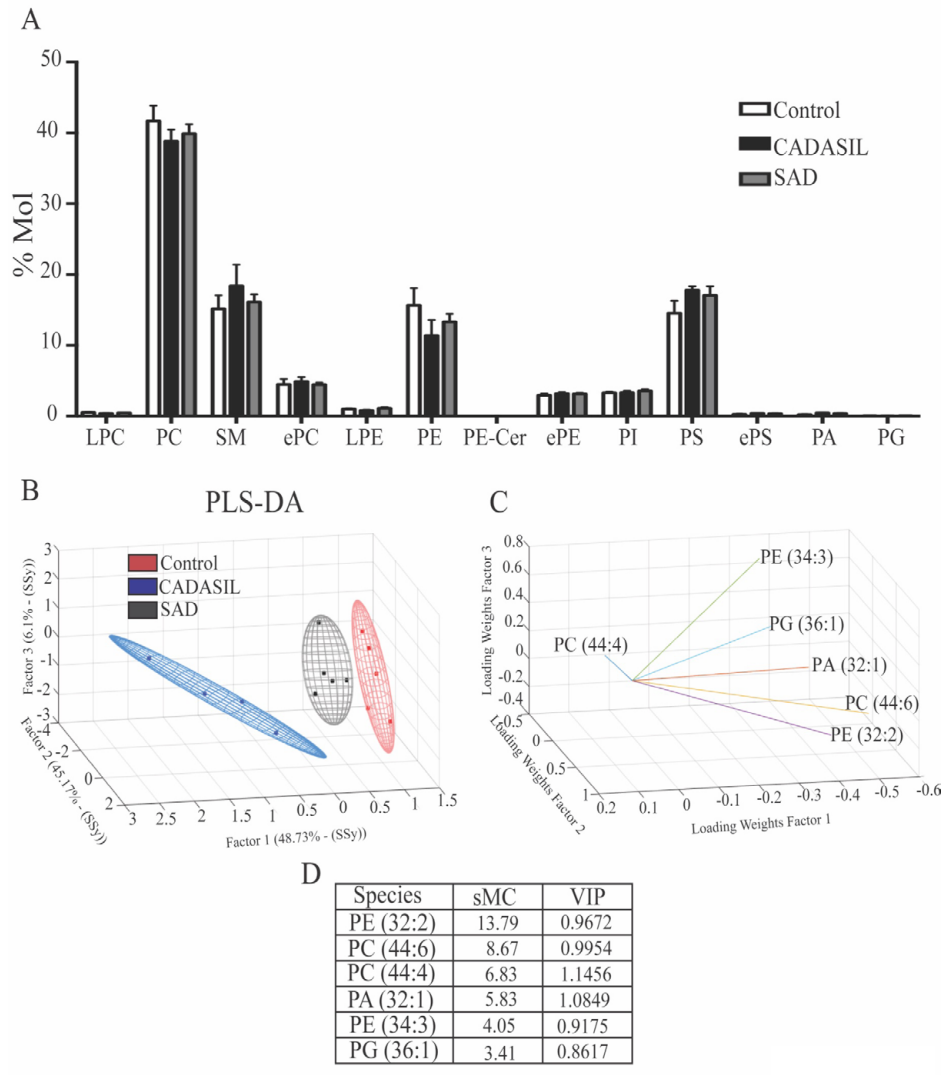
Phosphatidylserine (PS) is localized in the plasma membrane, solely in the cytoplasmic leaflet, where it forms part of protein docking sites necessary to activate several key signaling pathways. In mammalian tissues, PS is synthesized from either PC or PE exclusively by Ca 2+-dependent reactions where the head group of the substrate PL is replaced by serine [42]. We detected that PS (44:7), comprising docosahexaenoic acid (DHA) 22:6 and erucic acid 22:1, was the species with more importance for separating the three groups, with the ability to distinguish between the dementia groups or between each one independently with respect to the control group. However, the main differences between the CADASIL and SAD groups concerned LPE (18:2) and PS (44:9). In all the scenarios, the dementia groups showed alterations in the family of phospholipids related to cell signaling and neurotransmission in the temporal cerebral cortex, such as PS, LPC, and PC (Fig. 3 B-C).

Phosphatidylethanolamine (16:1/16:1) shows convergent deficiency in the white matter of the CADASIL and SAD groups

The white matter lipid profile was detected for 13 lipid species: PS (34%), PC (26%), and SM (16%) showed higher % Mol in control samples, PC (29%), SM (18%) and PS (17%) showed higher % Mol in CADASIL samples, and PC (40%), PS (17%) and SM (16%) showed higher % Mol SAD samples (Fig. 4.A). However, when we compared the lipid families, we detected no changes by ANOVA. Considering the above results, a supervised analysis was performed by PLS-DA to classify the samples and highlight the discriminant features among the white matter of the CADASIL, SAD and control subjects. The score plot of the PLS-DA model (Fig. 4.B) shows the intergroup separation along the three axes. Additionally, the CADASIL and SAD groups presented a different distribution pattern, showing that the phospholipid profile was different for each disease. Factor 1 explained 49% of the total variance, followed by factor 2 with 45% and factor 3 with 6%. The most discriminant signals were selected according to the significance multivariate correlation (sMC) and variable importance in the projection (VIP), through which the six species showing the most change were confirmed for PE (32:2) (sMC: 13,79/VIP: 0,9671), PC (44:6) (sMC: 8,67/VIP: 0,9954), PC (44:4) (sMC: 6,83 VIP: 1.1456), PA (32:1) (sMC: 5,83/VIP: 1.0849), PE (34:3) (sMC: 4,05/VIP: 0,9175) and PG (36:1) (sMC: 3,41/VIP: 0,8617), as observed in Fig. 4 C-D.

Phosphatidylethanolamine (16:1/16:1) has a convergent deficiency in the white matter of CADASIL and SAD. Phosphatidylethanolamine (PE) is the second most abundant phospholipid in mammalian cells. It is enriched in the inner leaflet of membranes and is

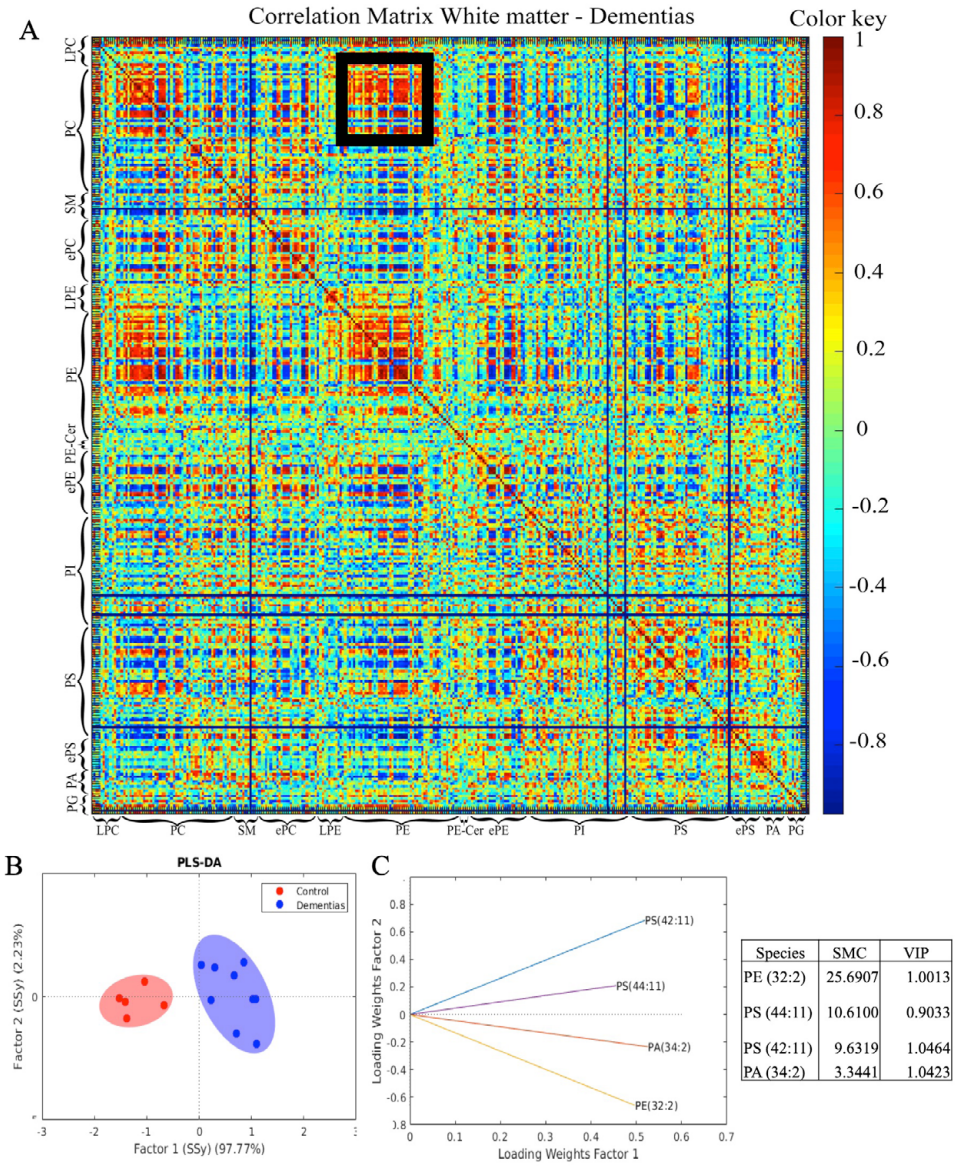
Transversal phenomenon in the brain parenchyma of dementias: Phospholipid profile analysis between CADASIL and Sporadic Alzheimer disease



Species	sMC	VIP
PE (32:2)	13.79	0.9672
PC (44:6)	8.67	0.9954
PC (44:4)	6.83	1.1456
PA (32:1)	5.83	1.0849
PE (34:3)	4.05	0.9175
PG (36:1)	3.41	0.8617

Figure 4. Lipid profile of the white matter from human CADASIL, SAD, and healthy subjects. A) The lipid class profiles are expressed as % Mol composition. All lipid species were measured (means), and the error bars represent the SEM. B) PLS-DA analysis of the lipid profiles from the temporal cortex of CADASIL, SAD, and healthy subjects. The panel show the factor score plots for PLS-DA and C) show the projections of the data with the sMC and VIP metrics. D) Values from sMC and VIP in a table. LPC, Lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; ePC ether phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PE-Cer, Phosphatidylethanolamine Ceramide; ePE, ether phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; ePS, ether phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol. n =4-5per group.

specifically abundant in the inner mitochondrial membrane. PE plays a main role as lipid chaperone, supporting the folding of certain membrane proteins; PE is needed for the activity of several respiratory complexes [60] and is a critical constituent of myelin [25]. We detected by PLS-DA that PE (32:2) is the more relevant species to separate the three study groups. PE comprising two chains of 16:1 (palmitoleic acid) was decreased in the CADASIL



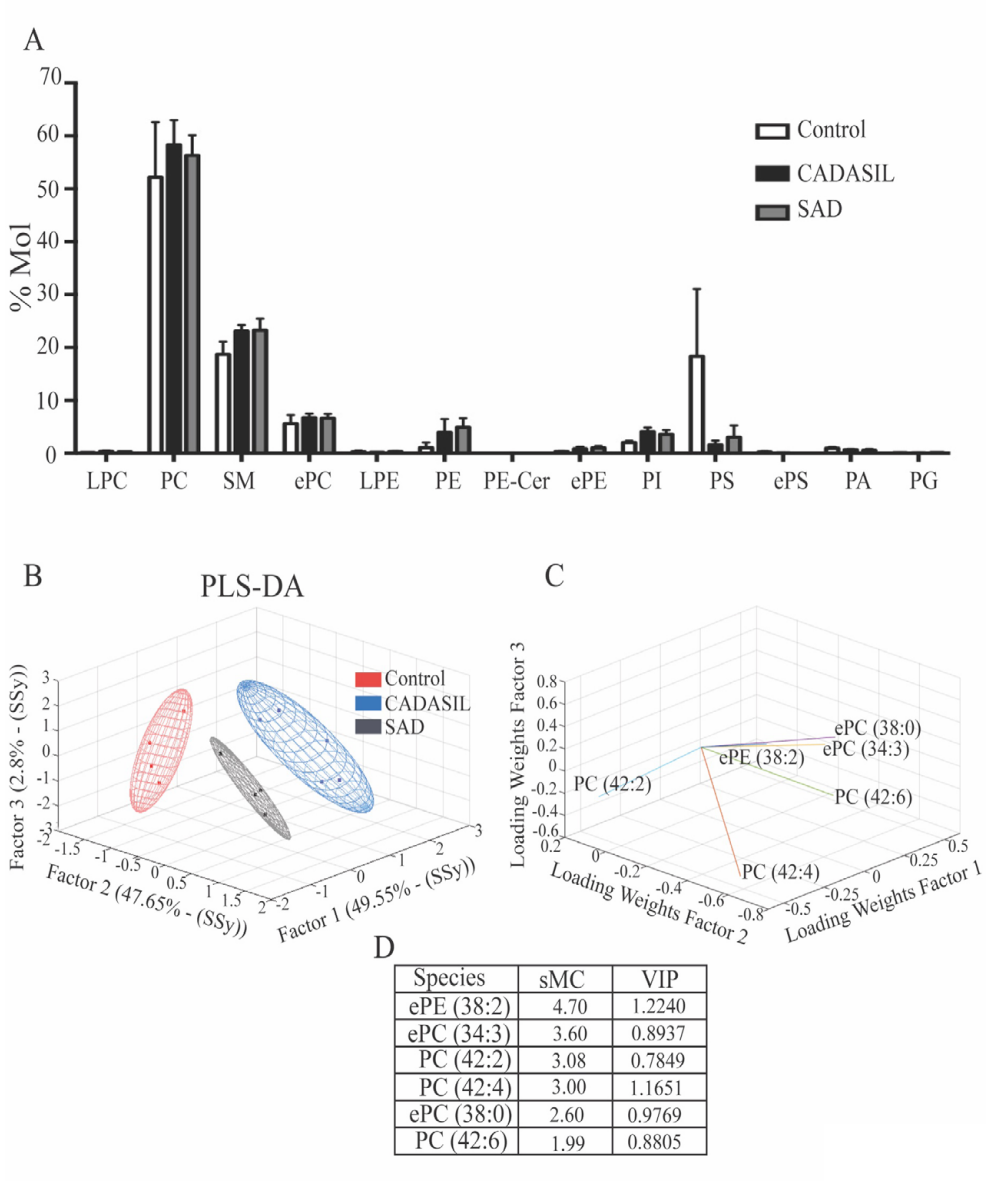
Species	SMC	VIP
PE (32:2)	25.6907	1.0013
PS (44:11)	10.6100	0.9033
PS (42:11)	9.6319	1.0464
PA (34:2)	3.3441	1.0423

Figure 5. Correlation matrix in Dementias from White matter. A) Heat map of the Pearson's linear correlation among the 352 measured metabolites. Metabolites are clustered using subspecies per families for CADASIL and SAD. B) PLS-DA analysis of the lipid profiles from dementia groups (CADASIL & SAD) with control group and C) show the projections of the data with the sMC and VIP metrics. n = 5 per group. LPC, Lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; ePC ether phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PE-Cer, Phosphatidylethanolamine Ceramide; ePE, ether phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; ePS, ether phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol. Black square: higher correlations between PE with some species of PC and LPC

and SAD groups compared with that in the control group. Similarly, we detected that PE (34:3) comprising 16:1 and 18:2 (Linoleic acid) shows differences in the dementia groups compared with that in the control (Fig. 4.D). Accordingly, a heat map for the correlation between CADASIL and SAD in the white matter showed a high divergence in these diseases. Nevertheless, we observed a high correlation among the PE species as well as for PE with some species of PC and LPC (Fig. 5.A). Interestingly, the PE correlationship was supported by PLS-DA, considering both dementias in one group (SAD & CADASIL) compared with the controls. We observed ellipsoids with a similar distribution pattern between dementias and different quadrants compared with the control. This finding was explained by the first component in 97,77% and the second one in 2,23% (Fig. 5.B), and the four subspecies more discriminant of dementias compared with the control were as follows: PE (32:2), PS (44:11), PS (44:12) and PA (34:2) (Fig. 5.C).

Phospholipidic differential pattern in the cerebrospinal fluid profile from CADASIL and sporadic Alzheimer's disease brains compared with healthy brains

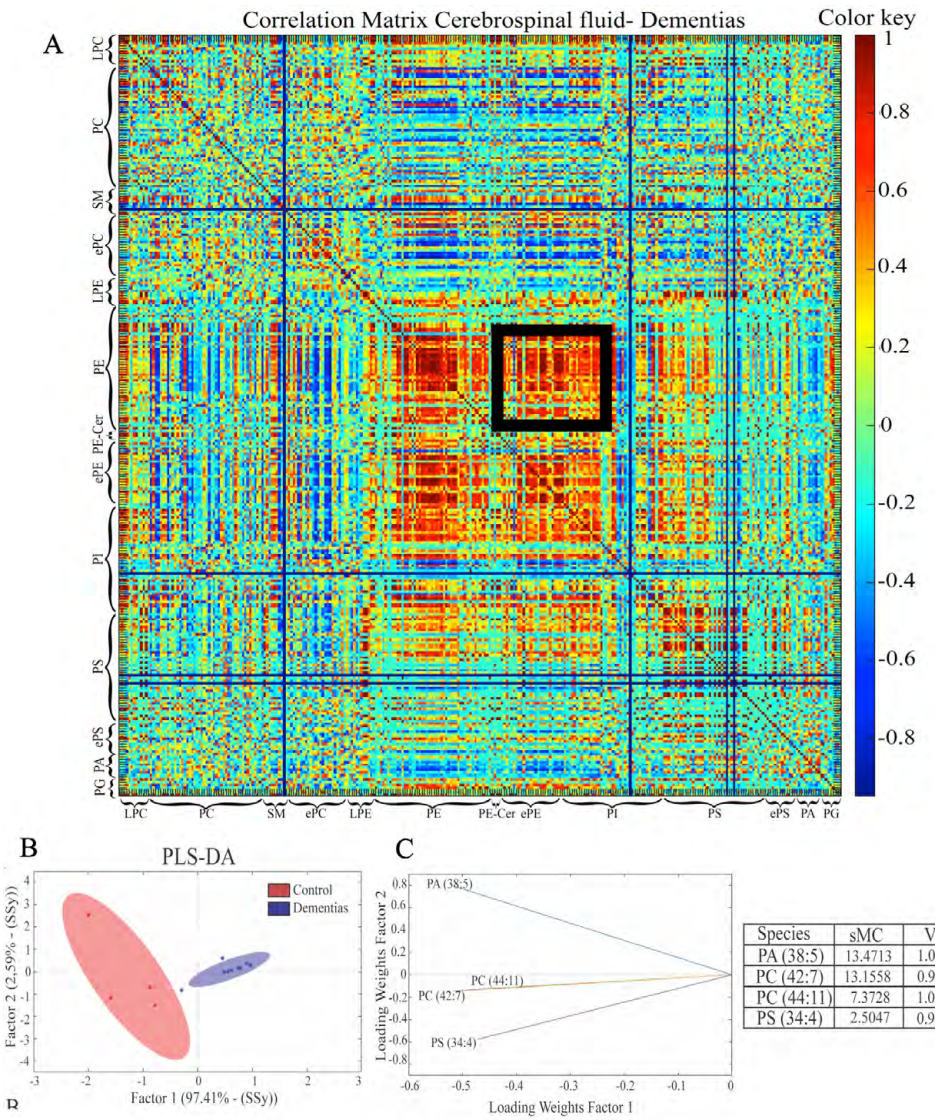
The cerebrospinal fluid is a clear colorless liquid that fills the subarachnoid spaces surrounding the brain, spinal cord, and ventricles. It is a metabolically active substance with bidirectional flow involved in the diffusion of metabolites in and out of the brain. Alterations in the composition of the cerebrospinal fluid markedly influence the functions of the central nervous system [(17)]. We investigated the lipid pattern in the CSF to identify relevant clinical biomarkers of CADASIL and SAD. The cerebrospinal fluid lipid profile revealed 13 lipid species: the % Mol values in the control samples were PC (52%), PS (18%), and SM (18%), those in the CADASIL samples were higher, with values of PC (58%), SM (23%) and ePC (7%), and those values in the SAD samples were PC (56%), SM (23%) and ePC (7%). This information is visualized in Fig. 6A, and a clear increasing tendency can be observed for PE and ePE in both dementias. The PLS-DA model built with our data set provided a good separation among the CADASIL, SAD and control subjects (Fig. 6.B), demonstrating the potential of cerebrospinal fluid PLs as biomarkers for dementias. Factor 1 explained 49,55% of the total variance, followed by factor 2 with 47,65% and factor 3 with 2,80%. Thus, numerous altered PLs were identified using the VIP as well as the sMC metric, where the six more changing species were confirmed as ePE (38:2) (sMC: 4,7/VIP: 1,2240), ePC (34:3) (sMC: 3,6/VIP: 0,8937), PC (42:2) (sMC: 3,08/VIP: 0.7849), PC (42:4) (sMC: 3,00/ VIP: 1.1651), ePC (38:0) (sMC: 2,6/VIP: 0,9769) and PC (42:6) (sMC: 1,99/VIP: 0,8805), as observed in Fig. 6 C, D. Furthermore, among the lipids highlighted by the heat map in Fig. 7.A, we observed an interesting correlation between some species of PE with ePE and PI that could be related to the tendency of these three species to increase in the dementia groups, as shown in Fig. 6.A. In general, ether lipids are peroxisome-derived glycerophospholipids, in which the hydrocarbon chain at the sn-1 position of the glycerol backbone is attached by an ether bond, and are important for the organization and stability of lipid raft microdomains involved in cellular signaling [(19)].



D

Species	sMC	VIP
ePE (38:2)	4.70	1.2240
ePC (34:3)	3.60	0.8937
PC (42:2)	3.08	0.7849
PC (42:4)	3.00	1.1651
ePC (38:0)	2.60	0.9769
PC (42:6)	1.99	0.8805

Figure 6. Phospholipid profile in the cerebrospinal fluid from human CADASIL, SAD, and healthy subjects. A) The lipid class profiles are expressed as % Mol composition. All lipid species were measured (means), and the error bars represent the SEM. B) PLS-DA analysis of the lipid profiles from the temporal cortex of CADASIL, SAD, and healthy subjects. The panel show the factor score plots for PLS-DA and C) show the projections of the data with the sMC and VIP metrics. D) Values from sMC and VIP in a table. LPC, Lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; ePC ether phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PE-Cer, Phosphatidylethanolamine Ceramide; ePE, ether phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; ePS, ether phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol. n =4-5 per group.



Species	sMC	VIP
PA (38:5)	13.4713	1.0545
PC (42:7)	13.1558	0.9703
PC (44:11)	7.3728	1.0228
PS (34:4)	2.5047	0.9490

Figure 7. Correlation matrix in dementias from Cerebrospinal fluid. A) Heat map of the Pearson's linear correlation among the 352 measured metabolites. Metabolites are clustered using subspecies per families for CADASIL and SAD. B) PLS-DA analysis of the lipid profiles from dementia groups (CADASIL & SAD) with control group and C) show the projections of the data with the sMC and VIP metrics. n = 5 per group. LPC, Lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; ePC ether phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PE-Cer, Phosphatidylethanolamine Ceramide; ePE, ether phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; ePS, ether phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol. Black square: higher correlations between PE with some species of PE with ePE and PI.

PA/PS downregulation and PC upregulation as common changes in the CSF of the CADASIL and sporadic Alzheimer's disease groups

Common changes in the sublipidic species between both dementias with respect to the control—PA (38:5), PC (42:7, 44:11)—were detected by PLS-DA (Fig. 7.B-C). Phosphatidic acid (PA) is a minor component of biological membranes (approximately 1% of PLs). PA is a central element in the synthesis and turnover of glycerophospholipids and is essential in numerous cellular functions, such as vesicular trafficking, signal transduction, cytoskeletal organization and cell proliferation [87]. Interestingly, in the general overview, PLS-DA did not show species such as PA. However, when we performed individual analysis of the dementia group compared with the control group, the sMC for PA (38:5) was 13,47, comprising oleic (18:1) and arachidonic acid (20:4) and PC (42:7), thus differentiating the dementia groups from the control group (Fig. 7B-C). Taken together, our data showed that PLs of the CSF differ between healthy people and those with CADASIL and SAD, mainly with regard to PA, as confirmed by pro-inflammatory fatty acids.

Discussion

The current study compares, for the first time, alterations in the phospholipid profile of individuals with CADASIL carrying Notch3 mutations with those with sporadic Alzheimer´s disease (SAD) and healthy brains to define similarities and differences in the phospholipid profile as a potential guide to develop biomarkers. In general, we found a similar disbalance in the dementia group (SAD & CADASIL), with a high correlation pattern identified by heat map analysis; further, a trend of increasing PC and PE and decreasing PS in the GM temporal cortex was found that was similar to the trends identified in the lipidic species detected in the CSF and was clearly discriminated by PLS-DA in comparison with the controls. Additionally, an inverse relationship of lipidic alterations (PC, PE reduction and PS increase) was observed in the white matter (corpus callosum) of both dementia groups (hypothetical scheme, Fig. 8) that could be supported by PS compensation in the central nervous system due to the PS, PC and PE triangular interdependency, the enzyme balance of which could solve the cell homeostasis dialogue between the endoplasmic reticulum and mitochondria [77, 78, 80].

Additionally, our findings showed significance in the multivariate correlation index (sMC), revealing that PLs such as PS 44:7 (18:1/22:6) in gray matter (GM), PE 32:2 (16:1/16:1) in white matter (WM), and PA 38:5 (20:4/18:1) in the cerebrospinal fluid (CSF) are common sublipidic species in CADASIL and SAD. These PLs function as neurotransmitter precursors (PS 44:7), myelin composition alterations (PE 32:2) and the inflammation response (PA 38:5), as supported by dendrite disruption and microgliosis in both CADASIL and SAD when compared with healthy brains. Those results point to a transversal phenomenon affecting the brain parenchyma in dementias, independent of the etiopathogenesis, possibly providing a common prodromal biomarker of dementia with potential vascular implications, supported by claudin-5 and vimentin labeling changes and suggesting parenchymal disruption in

Transversal phenomenon in the brain parenchyma of dementias: Phospholipid profile analysis between CADASIL and Sporadic Alzheimer disease

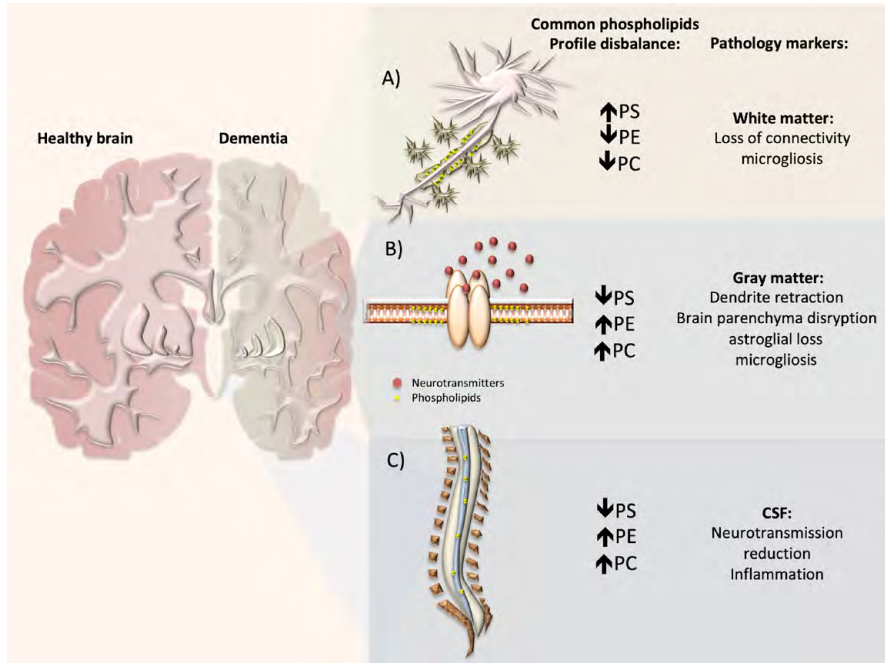


Figure 8. Hypothetical scheme of a common inverse relationship of phospholipidic profile in GM/CSF respect to WM in CADASIL and SAD. Main changes of lipid profile and tissue relationship at the A) White matter (WM), B) Gray matter (GM) and C) Cerebrospinal fluid (CSF) in CADASIL and SAD.

dementia. Therefore, proposing lipidomic profiles and lipidomic signatures could be useful to expand the understanding of pathologic mechanisms in neurodegenerative diseases. Interestingly, in this study, we obtained a significant model for discriminating between CADASIL and SAD patients and controls subjects that can identify similarities between these two different dementias.

PLs are biologically important molecules that form cellular membranes and are involved in the behavior of membrane proteins, receptors, enzymes and ion channels. They are also involved in maintaining lipid asymmetry, a dynamic process required to maintain normal neural membrane functions, such as neuroplasticity and vesicular transport [23]. Different areas of the brain differ in phospholipid composition, and alterations of PL metabolism appears to have a multifactorial origin involving the overactivation of phospholipases, increased anabolism of lysophospholipids, peroxisomal dysfunction, imbalances in the levels of saturated/unsaturated fatty acids contained in the structure of PLs and oxidative stress [33].

The CADASIL group exhibited a significant reduction in the levels of LPC and increased levels of PC in the temporal cortex, whereas the SAD group has the same tendency but without significance because of the intrinsic variability of the group. In general, these results suggest an imbalance between phospholipid and lysophospholipids that is regulated

by phospholipases. Activation of these phospholipases causes hydrolysis of membrane PLs and the generation of second messengers associated with neurodegeneration. In AD, overactivation of phospholipase A 2 (PLA 2), which catalyzes the hydrolysis of the ester bonds to liberate fatty acids (FA) and lysophospholipids, has been described. This enzymatic stimulation leads to decreased total levels of PLs and accumulation of their degradation products [34]. Our results agree with the previous report by Villamil et al., 2018 where LPC 20:4 and 22:6 were decreased in AD brains; however, they did not show differences in the levels of PC as in the present SAD group [81].

PC is the glycerophospholipid most abundant in all mammalian cell types and subcellular organelles; in this PL, a choline is attached to the phosphate group, leading to a cationic behavior [75, 77]. No study has reported data for PC in CADASIL, while modulation in AD has not been consistent across different studies. A decrease in PCs in patients with AD has been reported previously in peripheral blood samples [33, 83], postmortem brain samples [38, 54, 56, 84] and animal models [36, 70]. A higher concentration of PC C36:6 was significantly associated with a decreased prevalence of dementia [46]. Gaudin et al. demonstrated that two PC species were drastically depleted in senile plaques using a microdissection step on human brain samples and a semitargeted approach by UPLC-ESI-MS/MS [28]. On the other hand, research in individuals carrying PSEN1 mutations responsible for autosomal dominant AD (ADAD) showed a positive correlation between PC species (34:6, 36:5, 40:6) and CSF tau [13]. Other researchers found no changes in the frontal cortex, parietal, and temporal region from AD patients [61, 82]. Alterations in PC and LPC can show differences in the plasma of AD and MCI patients compared with those in healthy controls. The ratio of PC to LPC could represent pathophysiological markers in AD [43], and, generally, in dementias, as reflected by the neurotransmitter precursor disbalance.

Interestingly, PA 38:5 (20:4/18:1) and PC 42:7 (20:4/22:3) are common lipids in CSF in the dementia group. Free fatty acid 20:4 (Arachidonic acid-AA) is directly involved in synaptic functions as a retrograde messenger and a neuromodulator of exocytosis. Additionally, a decrease in PA has been associated with synaptosome loss [8]. Furthermore, some studies have shown that free ARA contributes to AD progression through various mechanisms: such as tau phosphorylation and polymerization and pro-inflammatory eicosanoids, which participate in neuroinflammation [66, 73]. Therefore, it is important to determine the correlation between GM and CSF to confirm the potential of some PLs involved in neurotransmission failure and proinflammatory environment as biomarkers to identify patients at risk of AD progression.

The best discriminant lipids distinguishing CADASIL and SAD patients from controls were PS 44:7 and LPE 18:2 in GM, PE 32:2 and PC 44:6 n WM and ePE 38:2, ePC 34:3 in CSF. Except for PS, all the other species were intermediates or derivates of metabolism of PC and PE. The second most abundant phospholipid in mammalian membranes is PE, which is enriched in

Transversal phenomenon in the brain parenchyma of dementias: Phospholipid profile analysis between CADASIL and Sporadic Alzheimer disease

the mitochondrial inner membranes compared with other organelles [77]. Reduced levels of PE have been correlated with the severity of AD progression in serum [35] and brain tissue [44, 54]. PE has the ability to destabilize existing Aβ fibrils and prevent the formation of new fibrils, providing another mechanism by which PE loss facilitates AD pathology; however, a deficit in PE is not present in MCI, a previous stage of the disease [85]. Similarly, the incorporation of ether-linked alkyl chains in PL alters their physical properties, such as packing, and affects membrane dynamics, reducing the membrane fluidity and increasing the rigidity (19), which could be supported by our previous studies in which alteration in the LPE and ePE levels was associated with memory impairment in 3xTg-AD mice [81]. Additionally, PE has been described as the main component of axon membranes [79] and is associated with myelin production [27], suggesting that its alteration is a common pattern in these two dementias.

PS is an endogenously occurring phospholipid that is thought to play a key role in brain cell membrane fluidity, transmission of brain cell activity and neural information [31]. We observed that PS 42:7 is the main discriminant species in the GM of the CADASIL and SAD groups and was reduced compared with the controls. However, while Wood et al., 2015 reported that the levels of PS were unaltered in the CSF, GM or WM [85], other groups reported that PS levels gradually decreased with increasing age, affecting memory and cognitive ability; in AD cases, this reduction was higher compared with that in control patients [88]. Additionally, we observed that PS 42:7 (18:1/22:6), comprising oleic acid and DHA, and a lower plasma concentration of these and other fatty acids are associated with cognitive decline in both healthy elderly people as well as patients with AD[2, 64].

Postmortem neuropathological staining by MAP-2 showed dendrite retraction in GM, and this reduction was more drastic in the SAD group than in the C group. Microtubule-associated protein-2 (MAP-2) is distributed in the neuronal cytoplasm, especially in dendrites. MAP-2 participates in the assembly and stabilization of the cytoskeleton to maintain neuron dendritic growth with the microtubules of the cellular cytoskeleton [22, 65]. Our results agree with MAP2-absent immunolabeling in areas of the entorhinal cortex and hippocampus of AD brains that colocalized with BA plaques [18, 71]; MAP-2 is detached from the microtubules by being sequestered by hyperphosphorylated tau in the AD brain. The removal of MAP2 contributes to increased microtubule dynamics, thereby inducing aberrant remodeling of dendrites in AD [22]. However, in the CADASIL group, MAP was associated with damage in autophagy through the ERK/MAPK pathway. In this sense, MAP1 is colocalized with autophagy markers, such as light chain 3 (LC3), sequestosome 1/p62 and Caspase-3 [39, 40]. However, PS 44:7 levels and MAP2 disruption, with the detection of an aggregated pattern by Nissl staining, support a common neurotransmission and plasticity disorder in both dementias.

Microglia are the most abundant mononuclear phagocyte of the CNS, continually surveying their microenvironments in normal and diseased brains while providing immune

surveillance and activation in response to infection, noninfectious diseases, and injury [1, 16, 30]. Although microglial hyperactivation or dysfunction is a potential mechanism leading to neurodegenerative and neuroinflammatory diseases, the roles of microglia are still under debate [47]. We observed strong reactivity of Iba-1 in CADASIL; however, this finding was not reported in postmortem tissue but, rather, in an animal model (TgPAC-Notch3 mice), which reported that myelin degradation is associated with a weak microglial response [15]. This finding is supported by the common alteration of PE 32:2 in both dementias because PE is crucial to the connectivity of fibers in the white matter region [58], oligodendrocytes [20] and astrocytes [11]; this type of tissue is widely affected by microvessel disruption in different dementias [55]. In our study, microgliosis was also observed in the first layers of the temporal cortex in SAD and a diffused pattern was observed in the WM. The role of microglia activation in AD pathogenesis depends on the internal parenchyma compensation; thus, microglia activation can have both beneficial and detrimental effects. However, in chronic disease, microglia activity is maintained with proinflammatory properties, causing more neuronal loss [41].

In the same way, astrocytes are the most abundant glial cells in the brain and are responsible for brain homeostasis. Under pathological conditions, reactive astrocytes are ubiquitously detected throughout the CNS [68]. It has been described that activated microglia induce A1 astrocytes through secreting tumor necrosis factor (TNF), interleukin-1α (IL-1α) and complement component 1q (C1q) and lose many neuroprotective functions of astrocytes [48]. Reactive astrocytes are identified by increased expression of intermediate filament proteins such as glial fibrillary acidic protein (GFAP) and vimentin [14]. We observed strong reactivity of GFAP and Vimentin in the CADASIL group. Accordingly, Hase et al., 2018 showed an increase in plasmatodendritic astrocytes and a decrease in the percentage of normal-appearing astrocytes [40]. However, GFAP expression by qRT-PCR was significantly reduced in the frontal cortex and WM from CADASIL patients [10]. Fibroblasts and myoblasts from CADASIL did not present changes in the actin and vimentin networks compared with those from the control [3], and variable levels of vimentin were observed [21], suggesting the heterogeneity of results. In AD, hypertrophic reactive astrocytes accumulate in the vicinity of senile plaques and are often seen in postmortem human tissue [52] and in animal models of the disorder even when variability was found between the evaluated regions [57, 63]. However, our data suggest global astrocytic loss in chronic impairment, such as in sporadic Alzheimer´s disease.

Claudin-5 is a tight junction (TJ) protein in the blood-brain barrier (BBB) that regulates the integrity and permeability of the BBB [49]. It has been described that, in AD and vascular dementia (VD), all claudin isoforms—2,5, and 11—are significantly higher in the neurons of the frontal cortex. A differential pattern in astrocytes and oligodendrocytes was detected, and PCA analysis discriminated the expression profile of TJ proteins, clearly separating AD from the controls and from other vascular brain diseases, including CADASIL [62, 69]. Taken together, these findings suggest that the gliovascular unit of the brain parenchyma is

severely impaired in the CADASIL and SAD groups.

In summary, PLs are a complex metabolic network involved in the fundamental function of the brain, and our data suggest a common influence on neurotransmitter precursors, white matter composition and the inflammation response in GM, WM and CSF, respectively, as a transversal phenomenon of the brain parenchyma is likely occurring with dementias such as CADASIL and SAD (Fig 8). Therefore, single phospholipid changes are not sufficiently sensitive diagnostic biomarkers, but profiles of PL changes can reflect dishomeostasis. A general overview of lipid profile modifications could provide insight into prodromal biomarkers for dementia. Additionally, this knowledge could uncover novel strategies for early prevention and/or early intervention in dementias. However, further research is required to understand the role of PLs in the pathogenesis and progression of dementia.

Funding: This research was funded by Colciencias # 111577757084 (GPC-G) and was supported by Colciencias #111565740581 and (GPC-G) and Scholarship Colciencias call 647 (AMSG).

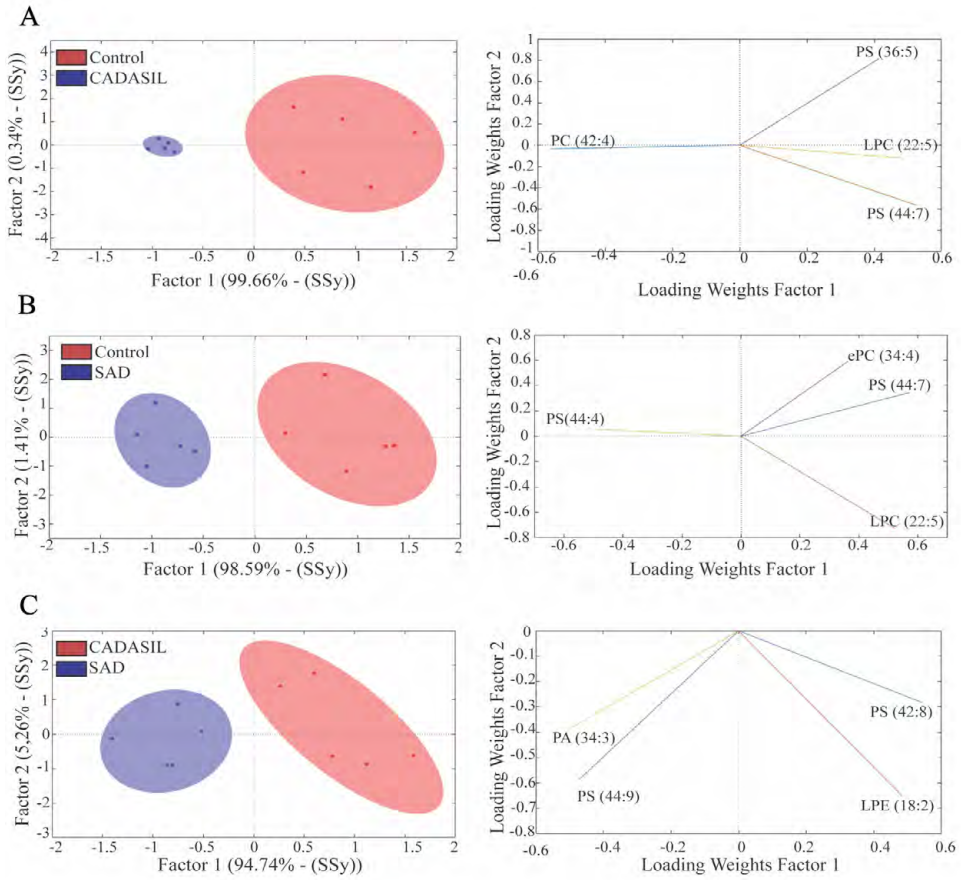
Acknowledgements

The authors thank the Cellular and Molecular Neurobiology Area and Brain Bank of the Neuroscience Group of Antioquia for providing scientific and technical support during the experiments. In particular, we thank Mariana Velez and Angela Barrera for their support in the extraction of lipids and collection of data for the patients. Lipid analysis was performed at the Kansas Lipidomics Research Center (KLRC).

Author's contributions

AMSG designed and realized the experiments, analyzed the data and wrote the paper; JDAL performed multivariate statistical analysis; JGV did immunostaining and quantification; DSF, MG and AVL revised and classified the clinical records from patients and advised on the neuropathological relevance of the findings; GPC-G designed, analyzed and interpreted the data, prepared the manuscript and provided critical revision. All authors read and approved the final manuscript.

Supplementary figures



Supplementary figure 1. Individual analysis of lipid profile of the temporal cortex from human CADASIL, SAD and control groups. A) PLS-DA analysis of the lipid profiles from CADASIL vs control group, showing the projections of the data with the sMC and VIP metrics. B) PLS-DA analysis of the lipid profiles from SAD vs control group showing the projections of the data with the sMC and VIP metrics. C) PLS-DA analysis of the lipid profiles from SAD vs CADASIL group showing the projections of the data with the sMC and VIP metrics. LPC, Lysophosphatidylcholine; PC, phosphatidylcholine; ePC ether phosphatidylcholine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; n= 5 per group.

References

1. Aguzzi A, Barres BA, Bennett ML (2013) Microglia: scapegoat, saboteur, or something else? Science.339(6116):156-61.

2. Amtul Z, Westaway D, Cechetto DF, Rozmahel RF (2011) Oleic acid ameliorates amyloidosis in cellular and mouse models of Alzheimer's disease. Brain Pathol.21(3):321-9.

3. Annunen-Rasila J, Karppa M, Finnila S, Yla-Outinen H, Veijola J, Tuominen H, Peltonen J, Majamaa K (2007) Cytoskeletal structure in cells harboring two mutations: R133C in NOTCH3 and 5650G>A in mitochondrial DNA. Mitochondrion.7(1-2):96-100.

4. Aureli M, Grassi S, Prioni S, Sonnino S, Prinetti A (2015) Lipid membrane domains in the brain. Biochim Biophys Acta.1851(8):1006-16.

5. Ballabio D, Todeschini R (2009) Multivariate Classification for Qualitative Analysis. Infrared Spectroscopy for Food Quality Analysis and Control.83-104.

6. Belkind-Gerson J, Carreon-Rodriguez A, Contreras-Ochoa CO, Estrada-Mondaca S, Parra-Cabrera MS (2008) Fatty acids and neurodevelopment. J Pediatr Gastroenterol Nutr.47 Suppl 1:S7-9.

7. Blennow K, de Leon MJ, Zetterberg H (2006) Alzheimer's disease. Lancet.368(9533):387-403.

8. Bothmer J, Markerink M, Jolles J (1992) Brain phosphatidic acid and polyphosphoinositide formation in a broken cell preparation: regional distribution and the effect of age. Neurochem Int.21(2):223-8.

9. Braak H, Braak E (1997) Staging of Alzheimer-related cortical destruction. Int Psychogeriatr.9 Suppl 1:257-61; discussion 69-72.

10. Brennan-Krohn T, Salloway S, Correia S, Dong M, de la Monte SM (2010) Glial vascular degeneration in CADASIL. J Alzheimers Dis.21(4):1393-402.

11. Camargo N, Goudriaan A, van Deijk AF, Otte WM, Brouwers JF, Lodder H, Gutmann DH, Nave KA, Dijkhuizen RM, Mansvelder HD, Chrast R, Smit AB, Verheijen MHG (2017) Oligodendroglial myelination requires astrocyte-derived lipids. PLoS Biol.15(5):e1002605.

12. Chabriat H, Joutel A, Dichgans M, Tournier-Lasserve E, Bousser MG (2009) Cadasil. Lancet Neurol.8(7):643-53.

13. Chatterjee P, Lim WL, Shui G, Gupta VB, James I, Fagan AM, Xiong C, Sohrabi HR, Taddei K, Brown BM, Benzinger T, Masters C, Snowden SG, Wenk MR, Bateman RJ, Morris JC, Martins RN (2016) Plasma Phospholipid and Sphingolipid Alterations in Presenilin1 Mutation Carriers: A Pilot Study. J Alzheimers Dis.50(3):887-94.

14. Chun H, Marriott I, Lee CJ, Cho H (2018) Elucidating the Interactive Roles of Glia in Alzheimer's Disease Using Established and Newly Developed Experimental Models. Front Neurol.9:797.

15. Cognat E, Cleophax S, Domenga-Denier V, Joutel A (2014) Early white matter changes in CADASIL: evidence of segmental intramyelinic oedema in a pre-clinical mouse model. Acta Neuropathol Commun.2:49.

16. Colonna M, Butovsky O (2017) Microglia Function in the Central Nervous System During Health and Neurodegeneration. Annu Rev Immunol.35:441-68.

17. Conly JM, Ronald AR (1983) Cerebrospinal fluid as a diagnostic body fluid. Am J Med.75(1B):102-8.

18. D'Andrea MR, Howanski RJ, Saller CF (2017) MAP2 IHC detection: a marker of antigenicity in CNS tissues. Biotech Histochem.92(5):363-73.

19. Dean JM, Lodhi IJ (2018) Structural and functional roles of ether lipids. Protein Cell.9(2):196-206.

20. Dimas P, Montani L, Pereira JA, Moreno D, Trotzmuller M, Gerber J, Semenkovich CF, Kofeler HC, Suter U (2019) CNS myelination and remyelination depend on fatty acid synthesis by oligodendrocytes. Elife.8.

21. Dong H, Ding H, Young K, Blaivas M, Christensen PJ, Wang MM (2013) Advanced intimal hyperplasia without luminal narrowing of leptomeningeal arteries in CADASIL. Stroke.44(5):1456-8.

22. Farah CA, Leclerc N (2008) HMWMAP2: new perspectives on a pathway to dendritic identity. Cell Motil Cytoskeleton.65(7):515-27.

23. Farooqui AA (2011) Lipid Mediators and Their Metabolism in the Brain.

24. Fillenbaum GG, van Belle G, Morris JC, Mohs RC, Mirra SS, Davis PC, Tariot PN, Silverman JM, Clark CM, Welsh-Bohmer KA, Heyman A (2008) Consortium to Establish a Registry for Alzheimer's Disease (CERAD): the first twenty years. Alzheimers Dement.4(2):96-109.

25. Fledrich R, Abdelaal T, Rasch L, Bansal V, Schutza V, Brugger B, Luchtenborg C, Prukop T, Stenzel J, Rahman RU, Hermes D, Ewers D, Mobius W, Ruhwedel T, Katona I, Weis J, Klein D, Martini R, Bruck W, Muller WC, Bonn S, Bechmann I, Nave KA, Stassart RM, Sereda MW (2018) Targeting myelin lipid metabolism as a potential therapeutic strategy in a model of CMT1A neuropathy. Nat Commun.9(1):3025.

26. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem.226(1):497-509.

27. Fressinaud C, Vallat JM, Rigaud M, Cassagne C, Labourdette G, Sarlieve LL (1990) Investigation of myelination in vitro: polar lipid content and fatty acid composition of myelinating oligodendrocytes in rat oligodendrocyte cultures. Neurochem Int.16(1):27-39.

28. Gaudin M, Panchal M, Auzeil N, Duyckaerts C, Brunelle A, Laprevote O, Touboul D (2012) Choline-containing phospholipids in microdissected human Alzheimer's disease brain senile plaque versus neuropil. Bioanalysis.4(17):2153-5159.

29. Geddes JW, Tekirian TL, Soultanian NS, Ashford JW, Davis DG, Markesbery WR (1997) Comparison of neuropathologic criteria for the diagnosis of Alzheimer's disease. Neurobiol Aging.18(4 Suppl):S99-105.

30. Ghosh S, Castillo E, Frias ES, Swanson RA (2018) Bioenergetic regulation of microglia. Glia.66(6):1200-12.

31. Glade MJ, Smith K (2015) Phosphatidylserine and the human brain. Nutrition.31(6):781-6.

32. Gong M, Rueschendorf F, Marx P, Schulz H, Kraft HG, Huebner N, Koennecke HC (2010) Clinical and genetic features in a family with CADASIL and high lipoprotein (a) values. J Neurol.257(8):1240-5.

33. Gonzalez-Dominguez R, Garcia-Barrera T, Gomez-Ariza JL (2014) Combination of metabolomic and phospholipid-profiling approaches for the study of Alzheimer's disease. J Proteomics.104:37-47.

34. Gonzalez-Dominguez R, Garcia-Barrera T, Gomez-Ariza JL (2014) Metabolomic study of lipids in serum for biomarker discovery in Alzheimer's disease using direct infusion mass spectrometry. J Pharm Biomed Anal.98:321-6.

35. Goodenowe DB, Cook LL, Liu J, Lu Y, Jayasinghe DA, Ahiahonu PW, Heath D, Yamazaki Y, Flax J, Krenitsky KF, Sparks DL, Lerner A, Friedland RP, Kudo T, Kamino K, Morihara T, Takeda M, Wood PL (2007) Peripheral ethanolamine plasmalogen deficiency: a logical causative factor in Alzheimer's disease and dementia. J Lipid Res.48(11):2485-98.

36. Grimm MO, Grosgen S, Riemenschneider M, Tanila H, Grimm HS, Hartmann T (2011) From brain to food: analysis of phosphatidylcholins, lyso-phosphatidylcholins and phosphatidylcholin-plasmalogens derivates in Alzheimer's disease human post mortem brains and mice model via mass spectrometry. J Chromatogr A.1218(42):7713-22.

37. Guey S, Mawet J, Herve D, Duering M, Godin O, Jouvent E, Opherk C, Alili N, Dichgans M, Chabriat H (2016) Prevalence and characteristics of migraine in CADASIL. Cephalalgia.36(11):1038-47.

38. Han X, Holtzman DM, McKeel DW, Jr. (2001) Plasmalogen deficiency in early Alzheimer's disease subjects and in animal models: molecular characterization using electrospray ionization mass spectrometry. J Neurochem.77(4):1168-80.

39. Hanemaaijer ES, Panahi M, Swaddiwudhipong N, Tikka S, Winblad B, Viitanen M, Piras A, Behbahani H (2018) Autophagy-lysosomal defect in human CADASIL vascular smooth muscle cells. Eur J Cell Biol.97(8):557-67.

40. Hase Y, Chen A, Bates LL, Craggs LJL, Yamamoto Y, Gemmell E, Oakley AE, Korolchuk VI, Kalaria RN (2018) Severe white matter astrocytopathy in CADASIL. Brain Pathol.28(6):832-43.

41. Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, Jacobs AH, Wyss-Coray T, Vitorica J, Ransohoff RM, Herrup K, Frautschy SA, Finsen B, Brown GC, Verkhratsky A, Yamanaka K, Koistinaho J, Latz E, Halle A, Petzold GC, Town T, Morgan D, Shinohara ML, Perry VH, Holmes C, Bazan NG, Brooks DJ, Hunot S, Joseph B, Deigendesch N, Garaschuk O, Boddeke E, Dinarello CA, Breitner JC, Cole GM, Golenbock DT, Kummer MP (2015) Neuroinflammation in Alzheimer's disease. Lancet Neurol.14(4):388-405.

42. Kim HY, Huang BX, Spector AA (2014) Phosphatidylserine in the brain: metabolism and function. Prog Lipid Res.56:1-18.

43. Klavins K, Koal T, Dallmann G, Marksteiner J, Kemmler G, Humpel C (2015) The ratio of phosphatidylcholines to lysophosphatidylcholines in plasma differentiates healthy controls from patients with Alzheimer's disease and mild cognitive impairment. Alzheimers Dement (Amst).1(3):295-302.

Transversal phenomenon in the brain parenchyma of dementias: Phospholipid profile analysis between CADASIL and Sporadic Alzheimer disease

44. Kosicek M, Hecimovic S (2013) Phospholipids and Alzheimer's disease: alterations, mechanisms and potential biomarkers. Int J Mol Sci.14(1):1310-22.

45. Lee LC, Liong CY, Jemain AA (2018) Partial least squares-discriminant analysis (PLS-DA) for classification of high-dimensional (HD) data: a review of contemporary practice strategies and knowledge gaps. Analyst.143(15):3526-39.

46. Li D, Misialek JR, Boerwinkle E, Gottesman RF, Sharrett AR, Mosley TH, Coresh J, Wruck LM, Knopman DS, Alonso A (2016) Plasma phospholipids and prevalence of mild cognitive impairment and/or dementia in the ARIC Neurocognitive Study (ARIC-NCS). Alzheimers Dement (Amst).3:73-82.

47. Li Q, Barres BA (2018) Microglia and macrophages in brain homeostasis and disease. Nat Rev Immunol.18(4):225-42.

48. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, Bennett ML, Munch AE, Chung WS, Peterson TC, Wilton DK, Frouin A, Napier BA, Panicker N, Kumar M, Buckwalter MS, Rowitch DH, Dawson VL, Dawson TM, Stevens B, Barres BA (2017) Neurotoxic reactive astrocytes are induced by activated microglia. Nature.541(7638):481-7.

49. Lv J, Hu W, Yang Z, Li T, Jiang S, Ma Z, Chen F, Yang Y (2018) Focusing on claudin-5: A promising candidate in the regulation of BBB to treat ischemic stroke. Prog Neurobiol.161:79-96.

50. Marchesi VT (2014) Alzheimer's disease and CADASIL are heritable, adult-onset dementias that both involve damaged small blood vessels. Cell Mol Life Sci.71(6):949-55.

51.McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Jr., Kawas CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R, Mohs RC, Morris JC, Rossor MN, Scheltens P, Carrillo MC, Thies B, Weintraub S, Phelps CH (2011) The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement.7(3):263-9.

52. Medeiros R, LaFerla FM (2013) Astrocytes: conductors of the Alzheimer disease neuroinflammatory symphony. Exp Neurol.239:133-8.

53.Mehmood T, Liland KH, Snipen L, Sæbø S (2012) A review of variable selection methods in Partial Least Squares Regression. Chemometrics and Intelligent Laboratory System.118:62-9.

54. Naudi A, Cabre R, Jove M, Ayala V, Gonzalo H, Portero-Otin M, Ferrer I, Pamplona R (2015) Lipidomics of human brain aging and Alzheimer's disease pathology. Int Rev Neurobiol.122:133-89.

55. Nelson AR, Sweeney MD, Sagare AP, Zlokovic BV (2016) Neurovascular dysfunction and neurodegeneration in dementia and Alzheimer's disease. Biochim Biophys Acta.1862(5):887-900.

56. Nitsch RM, Blusztajn JK, Pittas AG, Slack BE, Growdon JH, Wurtman RJ (1992) Evidence for a membrane defect in Alzheimer disease brain. Proc Natl Acad Sci U S A.89(5):1671-5.

57. Olabarria M, Noristani HN, Verkhratsky A, Rodriguez JJ (2010) Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. Glia.58(7):831-8.

58. Ousley AH, Morell P (1992) Individual molecular species of phosphatidylcholine and phosphatidylethanolamine in myelin turn over at different rates. J Biol Chem.267(15):10362-9.

59. Park JH, Jeon BH, Lee JS, Newhouse PA, Taylor WD, Boyd BD, Kim KW, Kim MD (2017) CADASIL as a Useful Medical Model and Genetic Form of Vascular Depression. Am J Geriatr Psychiatry.25(7):719-27.

60. Patel D, Witt SN (2017) Ethanolamine and Phosphatidylethanolamine: Partners in Health and Disease. Oxid Med Cell Longev.2017:4829180.

61. Prasad MR, Lovell MA, Yatin M, Dhillon H, Markesbery WR (1998) Regional membrane phospholipid alterations in Alzheimer's disease. Neurochem Res.23(1):81-8.

62. Romanitan MO, Popescu BO, Spulber S, Bajenaru O, Popescu LM, Winblad B, Bogdanovic N (2010) Altered expression of claudin family proteins in Alzheimer's disease and vascular dementia brains. J Cell Mol Med.14(5):1088-100.

63. Sabogal-Guaqueta AM, Osorio E, Cardona-Gomez GP (2016) Linalool reverses neuropathological and behavioral impairments in old triple transgenic Alzheimer's mice. Neuropharmacology.102:111-20.

64. Salem N, Jr., Vandal M, Calon F (2015) The benefit of docosahexaenoic acid for the adult brain in aging and dementia. Prostaglandins Leukot Essent Fatty Acids.92:15-22.

65. Sanchez C, Diaz-Nido J, Avila J (2000) Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. Prog Neurobiol.61(2):133-68.

66. Sanchez-Mejia RO, Mucke L (2010) Phospholipase A2 and arachidonic acid in Alzheimer's disease. Biochim Biophys Acta.1801(8):784-90.

67. Sastry PS (1985) Lipids of nervous tissue: composition and metabolism. Prog Lipid Res.24(2):69-176.

68. Sofroniew MV (2009) Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci.32(12):638-47.

69. Spulber S, Bogdanovic N, Romanitan MO, Bajenaru OA, Popescu BO (2012) Claudin expression profile separates Alzheimer's disease cases from normal aging and from vascular dementia cases. J Neurol Sci.322(1-2):184-6.

70. Tajima Y, Ishikawa M, Maekawa K, Murayama M, Senoo Y, Nishimaki-Mogami T, Nakanishi H, Ikeda K, Arita M, Taguchi R, Okuno A, Mikawa R, Niida S, Takikawa O, Saito Y (2013) Lipidomic analysis of brain tissues and plasma in a mouse model expressing mutated human amyloid precursor protein/tau for Alzheimer's disease. Lipids Health Dis.12:68.

71. Takahashi RH, Capetillo-Zarate E, Lin MT, Milner TA, Gouras GK (2013) Accumulation of intraneuronal beta-amyloid 42 peptides is associated with early changes in microtubule-associated protein 2 in neurites and synapses. PLoS One.8(1):e51965.

72. Tan RY, Markus HS (2016) CADASIL: Migraine, Encephalopathy, Stroke and Their Inter-Relationships. PLoS One.11(6):e0157613.

73. Thomas MH, Pelleieux S, Vitale N, Olivier JL (2016) Arachidonic acid in Alzheimer's disease. J Neurol Neuromedicine.1(9):1-6.

74. Tong M, Leao R, Vimbela GV, Yalcin EB, Kay J, Krotow A, de la Monte SM (2017) Altered temporal lobe white matter lipid ion profiles in an experimental model of sporadic Alzheimer's disease. Mol Cell Neurosci.82:23-34.

75. Touboul D, Gaudin M (2014) Lipidomics of Alzheimer's disease. Bioanalysis.6(4):541-61.

76. Tran T, Lee N, Buydens L, Blanchet L (2014) Interpretation of variable importance in Partial Least Squares with Significance Multivariate Correlation (sMC). Chemometrics and Intelligent Laboratory Systems.138:153-60.

77. van der Veen JN, Kennelly JP, Wan S, Vance JE, Vance DE, Jacobs RL (2017) The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. Biochim Biophys Acta Biomembr.1859(9 Pt B):1558-72.

78. Vance JE (2018) Historical perspective: phosphatidylserine and phosphatidylethanolamine from the 1800s to the present. J Lipid Res.59(6):923-44.

79. Vance JE, Pan D, Vance DE, Campenot RB (1991) Biosynthesis of membrane lipids in rat axons. J Cell Biol.115(4):1061-8.

80. Vance JE, Tasseva G (2013) Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells. Biochim Biophys Acta.1831(3):543-54.

81. Villamil-Ortiz JG, Barrera-Ocampo A, Arias-Londono JD, Villegas A, Lopera F, Cardona-Gomez GP (2018) Differential Pattern of Phospholipid Profile in the Temporal Cortex from E280A-Familiar and Sporadic Alzheimer's Disease Brains. J Alzheimers Dis.61(1):209-19.

82. Wells K, Farooqui AA, Liss L, Horrocks LA (1995) Neural membrane phospholipids in Alzheimer disease. Neurochem Res.20(11):1329-33.

83. Whiley L, Sen A, Heaton J, Proitsi P, Garcia-Gomez D, Leung R, Smith N, Thambisetty M, Kloszewska I, Mecocci P, Soininen H, Tsolaki M, Vellas B, Lovestone S, Legido-Quigley C, AddNeuroMed C (2014) Evidence of altered phosphatidylcholine metabolism in Alzheimer's disease. Neurobiol Aging.35(2):271-8.

84. Wong MW, Braidy N, Poljak A, Sachdev PS (2017) The application of lipidomics to biomarker research and pathomechanisms in Alzheimer's disease. Curr Opin Psychiatry.30(2):136-44.

85. Wood PL, Barnette BL, Kaye JA, Quinn JF, Woltjer RL (2015) Non-targeted lipidomics of CSF and frontal cortex grey and white matter in control, mild cognitive impairment, and Alzheimer's disease subjects. Acta Neuropsychiatr.27(5):270-8.

86. Xiang Y, Lam SM, Shui G (2015) What can lipidomics tell us about the pathogenesis of Alzheimer disease? Biol Chem.396(12):1281-91.

87. Zegarlinska J, Piascik M, Sikorski AF, Czogalla A (2018) Phosphatidic acid - a simple phospholipid with multiple faces. Acta Biochim Pol.65(2):163-71.

88. Zhang YY, Yang LQ, Guo LM (2015) Effect of phosphatidylserine on memory in patients and rats with Alzheimer's disease. Genet Mol Res.14(3):9325-33.

Transversal phenomenon in the brain parenchyma of dementias: Phospholipid profile analysis between CADASIL and Sporadic Alzheimer disease

89. Zhou Z, Marepally SR, Nune DS, Pallakollu P, Ragan G, Roth MR, Wang L, Lushington GH, Visvanathan M, Welti R (2011) LipidomeDB data calculation environment: online processing of direct-infusion mass spectral data for lipid profiles. Lipids.46(9):879-84.

90. Zhu S, Nahas SJ (2016) CADASIL: Imaging Characteristics and Clinical Correlation. Curr Pain Headache Rep.20(10):57.

CHAPTER 7

Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

Angélica María Sabogal-Guáqueta[1-3*], Vítor Passos de Pádua[1], Alejandro Marmolejo Garza[1-2], Erik Boddeke[2], Amalia Dolga[1*]

[1.]Department of Molecular Pharmacology, Faculty of Science and Engineering, Groningen Research Institute of Pharmacy, Behavioral and Cognitive Neurosciences (BCN), University of Groningen, Groningen, the Netherlands.

[2.]Biomedical Sciences of Cells & Systems, Molecular Neurobiology, Faculty of Medical Sciences, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands.

[3.]Neuroscience Group of Antioquia, Cellular and Molecular Neurobiology Area-School of Medicine, SIU, University of Antioquia, Medellín, Colombia

Manuscript in preparation

Abstract

The main immune surveillance cell of the brain, microglia, regulate synaptic pruning during development and induce or modulate inflammation during aging or chronic diseases. There is an increasing interest in physiological and pathophysiological functions of microglia, because they are sensitive to brain injury and disease, altering their phenotype and function to adopt a so-called activated state in response to any perceived threat to CNS integrity. They sense environmental changes, migrate towards the injury site, respond by releasing various factors and phagocyte microbes, dead cells, redundant synapses, protein aggregates, and cellular debris. Microglia, as the primary source of pro and anti-inflammatory cytokines or chemokines, are essential mediators of neuro-inflammation, being responsible of a large spectrum of cellular responses. Here, we show an overview of the role of microglia in human neurodegenerative diseases and provide an update on the current model systems to study microglia, including cell lines, iPSC-derived microglia, assembly or integration into 3D brain assembloids. We present various strategies to model and study their role in neurodegeneration, that provide a relevant platform for development of novel and more effective therapies.

Keywords: Microglia, organoids, neurodegenerative diseases

Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

Introduction

Microglia are the most important immune cells of the central nervous system (CNS), being the first line of immune defense. They were first identified by Pio Del Río Hortega in 1919 and described as cells with phagocytic capacity in brain tissue [1]. Microglia represent 0.5%–16.6% of the total number of cells in the human brain [2], highly depending on the brain region. Their origin during development has been under constant debate and it had been thought that microglia had the same origin as the hematopoietic stem cells (HSC), but nowadays it is widely accepted that the brain's immune cells derive from the early myeloid progenitors in the embryonic yolk sac (YS) at embryonic day 8.5, and are ontogenetically distinct from peripheral macrophages [3,4]. During embryonic development, these myeloid progenitor cells migrate via neural tube, colonize the entire parenchyma, and become microglia progenitors [5,6], which will mature to microglia cells. After birth, brain microglia already exhibit their definitive local density, following a wave of microglial changes and proliferation. During an organism's lifetime, the microglial population is long-lived and maintained by a high proliferation rate that accounts for their self-renewal, similar to the behavior of peripheral tissue-resident macrophages, such as lung macrophages. Whether infiltration of peripheral monocytes in the brain during the adulthood contributes to the total number of adult brain microglia is not clearly known. However, recent studies suggest that intrinsic apoptosis and self-renewal by several proliferation cycles maintain a relatively steady number in our brain microglia without any contribution of monocyte infiltration [7]. Microglia function as the brain sentinels by constantly scanning their environment for cell debris, possible infectious agents, and misfolded plaques that reside in the brain of patients suffering of neurodegenerative disorders. The mature cells have a pivotal role in physiological and pathological conditions, mainly in aging and age-related neurodegenerative diseases, but also in other conditions such as brain infections and psychiatric disorders [8–10].

Lessons from microglial ontogeny and its destitution

Most of the cells that comprise the brain parenchyma come from the neuro-ectoderm. However, microglia have a crucially different developmental origin. These developmental differences are intimately related to their function in embryonic and adult life [11]. It is reasonable to think that because microglia are tissue-resident macrophages of the CNS, they share most of their developmental signature with other tissue-resident macrophages [12]. Nevertheless, developmental studies have demonstrated that within the mesoderm-derived myeloid cells, microglia have still a different ontogeny. In this section, we will comprehensively delineate microglial development.

In mice, tissue-resident macrophages arise from erythromyeloid progenitors (EMPs) in two waves of production: an early primitive in the extra-embryonic yolk sac (YS) and a transient definitive, before the establishment of definitive hematopoiesis in the fetal liver and later in

the adult bone marrow [3,13–16]. Microglial origins can be traced to the primitive hematopoietic wave of early EMPs at embryonic day 7.5 (E7.5) in the YS [17]. This process is dependent on the transcription factors *Spf1* and *Irf8* and on colony-stimulating factor 1 (Csfr1) signaling [4,18]. Primitive macrophages from this first wave spread via the embryonic bloodstream and colonize the neuro-epithelium as early as E9.5 [4]. Conversely, other tissue-resident macrophages primarily develop from the transient definitive production wave of EMPs from the YS that colonize the fetal liver from E10 onward and mature into tissue macrophages through a monocytic intermediate[4,17]. Definitive hematopoiesis depends on the transcription factor *Myb*[17]. Genome-wide transcriptome and epigenome studies of mouse microglia showed that microglia cluster very different from other tissue macrophages and other glial cells [19–23]. However, there is no clear understanding of mechanisms underlying this dichotomy between macrophages and microglia.

The brain is an immune-privileged organ, with a blood-brain-barrier (BBB) which prevents peripheral macrophages and immune cells from infiltrating into brain parenchyma. Self-renewing resident macrophage cell should migrate there in earlier developmental stages, prior to the establishment of the BBB at E10 [24]. As the brain develops, microglia must undergo changes in function to support neurogenesis and synapsis pruning. An important transcriptomic and epigenome study demonstrated that microglia cluster in three main groups: early microglia (until E14), pre-microglia (E14 to few weeks after birth) and adult microglia (few weeks after birth onward) [25]. Each of these phases had unique regulatory elements. For instance, disruption of adult-specific transcription factor MAFB led to a disorder of microglial homeostasis [25]. Additionally, germ-free mice and offspring with antecedent of maternal immune activation exhibited dysregulation of adult-specific genes in early microglial stages, impacting microglial function, even demonstrating behavioral features. These findings support that the microglial developmental program is in synchrony with the developing brain and that genetic and environmental perturbations will affect brain homeostasis through microglia.

Most of microglial ontogeny studies have been performed in mice, making room for important debates about interspecies similarity[26] regarding CNS microenvironment, immune system evolutionary divergence, mouse models for neurodegenerative diseases. In human embryos, IBA1 positive microglia are present at gestational week 5.5 in the encephalon, and enter the brain through the ventricles [27]. All in all, the ontogeny of human microglia is a topic that remains to be studied in detail and its understanding is crucial for detection of strengths and limitations of murine findings.

Microglial depletion is a useful approach to study microglial biology *in vivo*. Absence of microglia in mice has been used to study repopulation capacity of microglia, as well as to interrogate fundamental mechanisms in neurodegeneration. Overall, there are two main approaches for microglial ablation: pharmacological and genetic interventions.

Homeostatic microglia express CSF-1 and survival of microglia depends on CSF-1R signaling [4,5]. An elegant proof-of-concept experiment in which a CSF-1R inhibitor that crosses the BBB was administered to mice that reported a yellow fluorescent protein (YFP) for microglia demonstrated that CSF-1R blockade effectively clears the microglial population [28]. Moreover, this clearance was harmless for mice. CSF-1R inhibition has been used to investigate microglia-dependent mechanisms in different neurodegenerative disorders. There are contradictory findings about the effects of microglia depletion in mice administered CSF-1R inhibitors, PLX5662 and PLX3397. For instance, PLX5662 exhibited neuroprotection and reduced leukocyte infiltration [29], whereas PLX3397 presented exacerbated neuro-inflammation [30]. PLX5662, which is more brain-penetrant that PLX3397, successfully depletes microglia [31]; and subsequent repopulation after depletion elicited anti-neuro-inflammatory effects, promoting brain recovery [32]. These findings suggest that after acute microglia depletion and repopulation may have beneficial effects in neurodegeneration. Another CSF-1R inhibitor, GW2850, depleted microglia and macrophages at the same time [33]. It may be that at early stages of development macrophages become sensitive to this drug, or that the treatment is not specific for CSF-1R and suppresses innate immune responses. Further research will interrogate ways to effectively deplete microglia with little side effects on other cell types employing the CSF-1R inhibition.

Hippocampal injections with liposomal clodronate, a bisphosphonate that induces apoptosis in phagocytic cell types, deplete Iba1 positive microglia *in vivo* [34]. Interestingly, a developmental role for microglia has been strengthened by different effects of acute depletion in adult mice [35], and in early post-natal life [36,37].

With the discovery of microglial-specific markers, genetic depletion of microglia has become attainable. However, mice undergo severe developmental defects and rarely survive to adulthood [21,38]. With the introduction of novel genetic techniques, microglia depletion in adulthood is attainable, more specific and efficient than pharmacological approaches. For instance, expression of the suicide gene herpes simplex virus thymidine kinase (HSVTK) and its mutant version under the CD11b promoter decrease inflammation [39–41]. HSVTK promotes apoptosis after administration of ganciclovir [41]. Another genetic approach is the administration of diphtheria toxin (DT) to transgene mice that express diphtheria toxin receptor by *Cre*-mediated recombination driven by the CX3CR1 promoter [8]. Both genetic methods reach up to 90% efficiency and are highly specific for microglia [42–44]. With these powerful approaches, it remains in the outlook to interrogate the importance of specific microglial subpopulations and developmental stages.

The role of microglia in the CNS

Under healthy physiological conditions, microglia plays an important role during prenatal development when they support neurons and axons to form prenatal connections [45]. During neurogenesis, microglia are able to phagocyte apoptotic neural stem cells [46]. Afterbirth,

microglia remain as the main responsible for removing not functional or redundant synapses, also called neural pruning [47–49]. On the other hand, they are essential in regulating the synapse strain and plasticity, by releasing different molecular signals, such as reactive oxygen species (ROS), nitric oxide (NO), neurotrophic factors and proinflammatory cytokines [50].

Microglia cells are crucial to CNS homeostasis. They protect neurons against NMDA-induced toxicity, and they are able to communicate with astrocytes to increase the effectiveness and to guarantee the most suitable microenvironment [51–53]. Furthermore, as the macrophages of the CNS, microglia can capture antigens via phagocytic and endocytic receptors, process antigens by the lysosomal machinery, express the major histocompatibility complex class II (MHC class II) and exhibit the peptides, as antigen-presenting cells [54]. Microglial activation leads to morphologic changes, which is one of the possible different ways to classify microglia. The "resting" microglia was described as a state that the microglia cells receive inhibitory signals from the CNS environment but still alert with their highly motile processes [21,55]. The "activated" microglia, has been related to a transformation in morphology in contact with foreign substances, releasing pro-inflammatory mediators as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) [56,57]. The "alternatively activated" microglial phenotype, has been characterized as a phagocytic and an anti-inflammatory morphology, releasing protective and trophic factors [58]. However, recent studies suggest that this classification may be ineffective because due to a wide spectrum of microglial phenotypes [59,60].

Neurodegenerative diseases and microglia

Microglial activation has been associated with several disorders, including neurodegenerative diseases such as Alzheimer Disease (AD), Parkinson Disease (PD), Amyotrophic Lateral Sclerosis (ALS) [61]. These diseases have different protein markers and symptoms, but they have in common a similar physiopathology: accumulation of misfolded proteins that entails intracellular inclusions and followed by neuronal death [62]. As microglia is responsible for CNS homeostasis and phagocytosis, they can become activated in the presence of misfolded proteins, and could initiate molecular pathways detrimental to survival or surrounding cells, such as neurons, by releasing cytotoxic and pro-inflammatory factors [63,64].

Alzheimer disease

Alzheimer Disease (AD) is characterized by progressive neuronal loss in brain regions responsible for learning and memory, being highly associated with dementia. AD represents around 50-75% of dementia patients. 95% of AD cases are sporadic cases, and just 5% is considered familial AD, but in both cases, there is a multifactorial etiology behind [65]. Mutations in ApoE genes are related to late AD development [66], while vascular risk factor is the most related modifiable risk [67]. AD normally occurs in elderly people, and it is very difficult to diagnose at the beginning of symptoms. However, the pathophysiology of AD is well known, and the major markers at the CNS are the beta-amyloid plaques (βA) and neurofibrillary tangles (NFT) [68]. In this scenario, microglial role is crucial at the early stages because they possess the ability to remove the amyloid aggregates. However, with aging, this microglial ability to clear up the debris or apoptotic neurons begins to decrease, in parallel to the inflammatory signals that are increasing. Changes in the cytokine profile and inflammatory markers were detected in brain microglia of post mortem patients, which showed high levels of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β [69,70]. These data were corroborated by observations derived from brain tissue from AD patients, where microglia (positive for Iba-1 marker) lose motility necessary to assist neurons and exhibit high expression of cytokines receptors. Oppositely, other microglial proteins (CD68, MSR-A), the role of which is clearance of damaged cellular material, are positively associated with AD and impaired cognitive function [71].

During aging, microglial capacity to clean up βA decreases, leading to βA accumulation, and inflammation, that in turn facilitates more βA formation and aggregation, leading to a vicious detrimental cycle. Consequently, the AD progression becomes unavoidable, and the sum of it all entails neuronal death, with the activation of caspase-3, 6 and 8 which initiate apoptosis [72]. The initial microglia activation is beneficial up to a limit when it starts to be harmful by releasing cytokines and facilitating further βA aggregation [73].

Parkinson disease

Parkinson Disease (PD) is a neurodegenerative disorder that affects 1-3% of the world population above 60 years [74]. There are two different types to develop PD: genetic heritage or familial and sporadic disease [75,76]. Therefore, multiple mechanisms lead to the same pathophysiology in the PD brain: the loss of dopaminergic neurons as an essential feature, together with α-synuclein aggregation [77].

In healthy physiological conditions, genes, such as protein declycase DJ-1, Phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1), Parkin, and Leucine Rich repeat kinase 2 (LRRK2), regulate microglia function - inflammation, surveillance and phagocytosis, and those genes are the most affected in PD [78,79]. These changes lead to an increase in the markers of inflammation and an increase in ROS associated with a loss of DJ-1 and PINK1 function and gain of Parkin and LRRK2 function [80–82]. There is no doubt about the crucial role of microglia in PD pathophysiology, by releasing more proinflammatory substances, which accelerate neuronal cell death. The understanding of PD pathology is hampered due to the disease complexity.

Amyotrophic lateral sclerosis

Amyotrophic Lateral Sclerosis (ALS), a motoneuron degeneration of the CNS, is a multifactorial disease, with 5-20% of cases having a hereditary component while the majority are sporadic cases [83]. The ALS physiopathology involves changes in various physiological pathways,

including increased oxidative stress, reduction of neurotrophic support, failure in protein homeostasis and RNA processing. In ALS patients, motoneuron degeneration has been associated with stimulation of excitotoxic pathways, glial inflammation [84], and microglial activation. Infiltrating lymphocytes at sites of motoneuron injury are highly correlated with the disease severity [85,86]. These processes contribute to the release of pro-inflammatory cytokines and chemokines, a decrease of neurotrophic factor expression and release, and an increase in the secretion of neurotoxic factors, that ultimately contribute to motoneuron cell death and neuronal network degeneration [87].

Huntington disease

Huntington's disease (HD), an autosomal dominant neurodegenerative disease is characterized by progressive motor dysfunction, cognitive impairment, and is accompanied in some cases by neuropsychiatric symptoms [88,89]. The mutant protein in HD, huntingtin (mHTT) results from expanded CAG repeats and contributes to the formation of a polyglutamine strand of variable length at the N-terminus [90]. Although the pathogenic mHTT is ubiquitously expressed in the CNS and also different types of neuronal cells, it causes a preferential damage and cell loss in the striatum, particularly affecting medium spiny neurons. As HD progresses, the atrophy of caudate and putamen expand to surrounding brain areas, reaching the cerebral cortex [91]. At the cellular level, mHTT proteins promote neuronal dysfunction and cell death through a number of molecular mechanisms, including disruption of cellular proteostasis, transcription and mitochondrial structural and functional alterations [92]. Marked astrogliosis and microgliosis were detected in post-mortem brains of HD patients, while in healthy brains these processes were absent [93]. It was reported that microglial activation and its associated structural alterations were present in all grades of HD patients' brains, and the structural alterations correlated to the degree of neuronal dysfunction [91]. Increased immune activation in the CNS and peripheral immune system in HD has been described by identifying increased IL-6, IL-8 and chemokines such as eotaxin-3, MIP-1β, eotaxin, MCP-1 and MCP-4 in plasma [94,95]. Monocytes isolated from HD gene carriers, which highly express mHTT, are pathologically hyperreactive in response to various stimuli, including lipopolysaccharide (LPS) stimulation [96]. Therefore, a hyperreactive immune system, together with microglial activation, have been recognized as an important feature of HD.

Other neurodegenerative diseases such as multiple sclerosis, and frontotemporal dementia, highlight the key role of microglia in both cellular homeostasis and neurological disorders that affect millions of people worldwide (Figure 1) [97,98]. In conclusion, better understanding of physiological and pathophysiological mechanisms in microglial biology is fundamental to elucidate ways to tackle progressive neurodegeneration.

Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

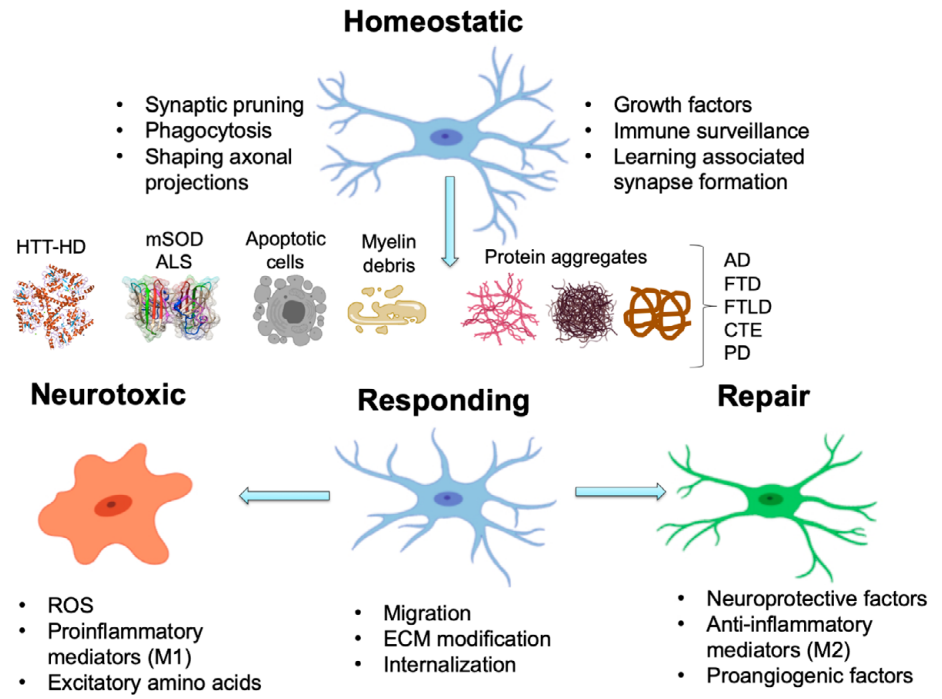


Figure 1. Microglia in neurodegenerative diseases. Microglia serve numerous functions in the brain, including synaptic pruning, phagocytosis, secretion of growth factors to maintain homeostasis, immune surveillance, shaping axonal projections, among others to control the homeostasis. Usually, microglia is activated from different stimulus, such as protein aggregates, myelin debris, apoptotic cells, etc. Its answer is described as reparative or toxic depending of the anti-inflammatory or proinflammatory factors secreted, respectively. AD: Alzheimer disease; ALS: Amyotrophic lateral sclerosis; CTE: Chronic traumatic encephalopathy; ECM = extracellular matrix; FTD: frontotemporal dementia; FTLD: frontotemporal lobar degeneration; HD: Huntington disease; HTT: huntingtin; PD: Parkinson disease; mSOD: superoxide dismutase 1; ROS = reactive oxygen species; TLR = toll-like receptor.

Models to study microglia function in the CNS

In vitro studies on microglial cells provide a good platform to understand fundamental questions on microglial biology under healthy and conditions modeling various brain pathologies. Currently, there are five main ways to culture microglia and perform experiments to learn more about the balance between the beneficial and detrimental role of microglia: cell lines (murine or human), primary microglia (mainly murine), stem cell-derived microglia (murine and human), organotypic brain slices of transgenic animals and brain spheroids/ organoids.

Cell lines

Cell lines are immortalized cells, collected and generated by treatment with oncogenes, that induce them to become immortalized, although cells might undergo spontaneous immortalization as well. However, immortalized cells can be very sensitive to differentiation,

which represent an inconvenience [56]. There are various cell lines generated from rat, mouse, macaque, and human. From rat, the most studied microglial-like cell line is HAPI [99] - highly aggressively proliferating immortalized - and it is considered the first cell line generated by unprompted immortalization, although the exact mutation that enables immortalization is not yet known. The most numerous protocols for microglial cell lines are generated from mouse, with the cell line BV2 being the most widely used. BV2 cell line was generated via *v-raf/v-myc* oncogenes [100]. These cells respond to LPS, are able to phagocyte and increase pro-inflammatory gene in response to LPS or various stimuli, mediate increases in ROS levels after exposure to βA fibrils and α-synuclein [101–103]. Meanwhile, HMO6 cells are derived from embryonic human primary microglia with a *v-myc* oncogene carrying PASK 1.2 retroviral vector [104].

These cell lines show distinctive microglial/macrophage cell markers and similar behavior (cytokine release, migration, and phagocytosis) in the presence of the gram-negative bacteria lipopolysaccharide (LPS). However, it has been described that IL-1B release and NO production in BV2 and HMO6 cells differs from primary microglia [101,105]. Likewise, Nagai and collaborators demonstrated important differences in protein profile and mRNA expression after βA induction between primary microglia and human cell lines. Protein profile and mRNA expression after LPS or βA induction were distinct from human microglia responsiveness [69]; Butovsky et al, showed that the treatment of N9 and BV2 cells with MCSF macrophage colony-stimulating factor) and TGF-β 1 did not induce the expression of microglial molecular pattern as the adult microglia. These studies underline the limitations of microglial cell lines in terms of molecular expression, morphology, proliferation, and adhesion [21].

Primary microglia

Primary microglia cultures can be extracted from non-human primate, rodent, or human brains. All three cultures have positive and negative features in relation to microglial function. Cells from rodents are easier to obtain because the animal's centers are accustomed to raising them. However, many unanswered questions remain about the use of rodents, concerning developmental, genetic, and physiological differences among human and mice. Studies have shown many divergences in mice and humans' embryonic development, particularly during gastrulation and organogenesis [106]. An extensive comparison in microglial gene expression of human and mice resulted in critical differences, principally in aging-related genes. Moreover, in uncommon cases, the same genotype could trigger divergent phenotypes in both rodents and humans [107–109].

On the other hand, in most studies, human brain tissue was derived from neonatal donors [110], and recent studies are making possible the use of adult's brain tissue [21]. Besides that, the birth and death conditions can be nowadays better controlled, which ensures superior cell preservation. Meantime, critical differences between microglia derived from rodents

Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

and humans, especially in the context of elderly, increase the need for new and relevant models to study neurodegenerative disorders and also microglial biology related to healthy aging [26,111].

Although non-human primates are evolutionarily closer to humans, and they are in specialized centers what allows control postmortem conditions of brain tissue, they are difficult to raise, and just few centers are able to handle them, therefore, primary microglia from them is not widely used [56]. On the other hand, primary microglia from humans started to be more commonly used and these microglia are considered one of the best option to study and understand the human microglial biology [112–114]. However, there are two principal technical features that hamper their study in age-related neurodegenerative diseases: i) the difficulty in obtaining healthy samples, since one can only obtain brain tissue from abortion, and this young microglia is quite distant and different from old, more mature microglia, or ii) from autopsies, where it is impossible to control antemortem conditions [115]; and the existing microglia phenotype, which can be altered by the delay in withdrawing the brain tissue. Considering these impediments and limitations, microglia extracted from autopsies of patients suffering from different neurodegenerative disorders could provide answers on their implication on the molecular pathways that link disease pathogenesis and the degree of brain degeneration [59,116].

Organotypic brain slices

The study of microglial function in neurodegenerative conditions presents various technical complications. First, the most accepted *in vivo* and *in vitro* models of neurodegenerative disease recapitulate key molecular phenotypes [117], but they do not accurately replicate disease progression and/or associated pathology (i.e. hyper-phosphorylated tau without neurofibrillary tangles, APP isoform influence AD progression, over-expression of tau impairs motor function and interferes with cognitive assessment) [117–119]. Second, efforts towards *ex vivo* approaches, such as primary microglia obtention, present technical culprits regarding the physiological state after dissociation of brain tissue and culture [120]. Third, the co-culture system of glial cells and neurons, which aims to mimic the complex brain microenvironment [121], does not accurately replicate it. Efforts to study microglial function without acutely affecting microglial microenvironment and interactions have risen, in particular with the use of organotypic brain slices [122].

An important feature of the organotypic brain slice culture system is that it preserves the cytoarchitecture of the brain, which is close to an *in vivo*-like situation. Moreover, the maintenance of the tridimensional structure and architecture of the brain preserves critical cellular interactions, making possible to perform dynamic profiling and long-term cellular tracking [123]. Working with brain slices reduce, refine and replace animals used and increase the capacity to perform medium-to-high throughput drug screenings [122,124]. Furthermore, brain slices can be obtained from embryonic, neonatal and adult specimens, highly valuable for neurodevelopmental stages and neurodegenerative conditions. Brain

slices are a valuable model for microglial research, while there are some considerations when employing brain slices: although they may be a good *in vivo* replacement, behavioral testing and correlation cannot be performed. Brain slices are axotomized, and there is an acute inflammatory response to cutting which is driven by astrocytes [125]. Microglia undergo morphology changes in the first days *in vitro* (DIV), but after 10 DIV, microglia return to an *in vivo*-like situation [126]. Lastly, they lack blood flow, limiting their viability. In spite of this, efforts to extend viability have led to up to 28 DIV [122] of life-time. All in all, brain slices may provide acceptable replacement for *in vivo* studies to elucidate microglial physiology.

In homeostasis, microglia support neurons by secreting neurotrophic factors, and clearing debris in a house-keeping manner [55,127]; in neurodegenerative disease, microglial function is profoundly impaired [128] perpetuating chronic neuroinflammation. These important interactions are not entirely recapitulated in cell culture systems. For instance, functional studies of isolated microglia are confounded by the disruption of the microenvironment[56]. The slice culture system is a well-established model [129–131] to study microglial function as close to as *in situ* as possible. A valuable tool to study microglial function is their depletion [132] in brain slices. Jung et al have successfully depleted microglia in brain of microglia/ macrophage reporter mice with the toxin clodronate [133], paving the way for microglial depletion in brain slices [134–137]. Hellwig and colleagues have demonstrated for the first time that microglia prevent amyloid burden in brain slices from wild-type mice by depleting them, strengthening their phagocytic role [136]. Following this approach, a phagocytic and chemotactic role for microglia in AD has been proposed by Daria and colleagues. In their study, they employed AD mice brain slices that were treated *ex vivo* with clodronate to deplete the endogenously expressed microglia. By replenishing the brain slices with either young or old microglia, the amyloid burden present in the brain slices was decreased and correlated with microglial recruitment to the plaque. Moreover, exposing old microglia to secreted factors of young microglia or supplementing the culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) could elicit functional recovery of old microglia and even reduce amyloid plaque size [124]. Overall, these findings indicate the critical role of microglia in cleaning protein aggregates and propose potential therapeutic approaches aimed to reinforce microglial phagocytosis to revert neurodegenerative disease pathology.

Induced Pluripotent Stem Cells (iPSC)

Stem cell biology is evolving every day. Microglia can be generated from two different types of stem cells: embryonic stem cells (ESC), derived from a blastocyst, and induced pluripotent stem cells (iPSC). Although ESC can be reprogrammed in any cell type, currently the majority of the protocols of human microglial generation are not employing ESC as a primary cell type. On the contrary, iPSC technology is widely used to differentiate and study microglial biology. The iPSC can be produced from adult human cells, which have undergone a reprogramming via overexpression of immature state-specific transcription factors (*OCT4*, *SOX2*, *KLF4*, and *c-MYC*) knowns as Yamanaka reprogramming factors [138]. With the help of these factors, the nullipotent, mature cell is able to convert its fate backwards into a pluripotent cell, embryonic-like state. iPSC technology was particularly innovative, as it allowed it for the

first time to evaluate the effects of a particular gene on familial monoallelic diseases as well as complex non-familial idiopathic diseases. The latter studies are performed using iPSC patient-derived cells, that are differentiated into a plethora of brain cells, including neurons, astrocytes, oligodendrocytes and recently microglia, systems that were previously not easily available for experimental investigation [139,140]. As a consequence, primary microglial cultures, organotypic brain tissue culture systems of transgenic animals and emerging iPSC-based microglia represent valuable experimental systems to study human familial neurodegenerative diseases and age-related non-familial neurodegenerative diseases [141,142].

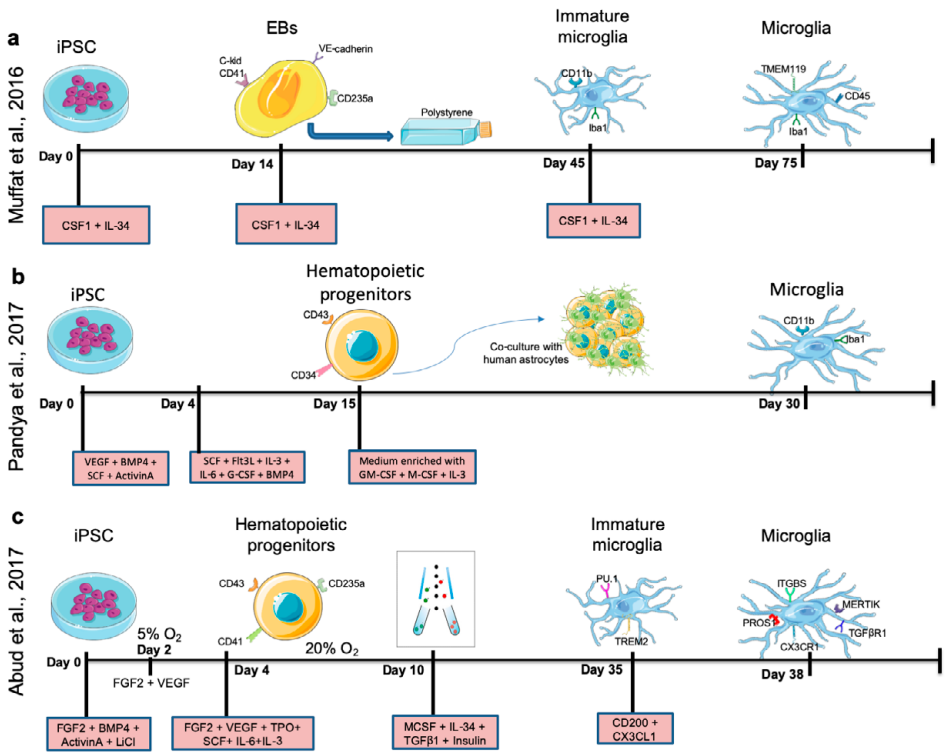
Recent studies have shown that human aging can be modeled across direct cell type conversion in any kind of cell and transcriptomic signatures of their donors age do not disappear after reprogramming protocols, indicating the importance of iPSC tools to study age-related diseases [143,144].Nonetheless, iPSC technology has its own limitations. It was suggested that residual epigenetic features from donors might sometimes persist in iPSC [145–147] and most cases of degenerative diseases have multifactorial risks, which is hard to mimic *in vitro* [144,148].

To reverse this scenario, new techniques are emerging to induce and even accelerate aging. iPSC-neurons were submitted to general stressors such as hydrogen peroxide, MG-132 and concanamycin [149,150] showing promising results; however more tests are necessary to secure their reproducibility, since cells showed differential vulnerability to various stressors. Other experiments performed for inducing an accelerated aging phenotype, a progerin-a protein involved in Hutchinson-Gilford progeria syndrome, was overexpressed that initiated age-related markers and cell death pathways in neurons [151,152].

Despite all this, iPSC-derived neurons and glial cells have been used to answer various questions related to their relevance to model human diseases and also their experimental practicality. Disease modeling and drug testing seem to be the mainstream end goal on using iPSC technologies, in addition to 3D models to study the CNS cell interactions that are generally difficult to mimic in a 2D *in vitro* monoculture [141,153].

Microglia differentiation protocols

Since the discovery of the exact origin of microglia, few protocols have been described to generate microglia from iPSC. Differentiation of iPSC to microglia is really new, and the first protocol appeared in 2016. The first author to publish was Muffat et al. 2016 [154], followed by Abud et al. 2017 [155], Douvaras et al. 2017 [156], Haenseler et al. 2017 [157], Pandya et al. 2017 [158] , Brownjohn et al. 2018 [159], Garcia et al. 2018 [160] and McQuade et al. 2018 [161] as illustrated in Figure 2. These reported protocols share similar patterns in the microglial differentiation steps, however the most common denominator is represented by colony-stimulating factor 1 (CSF1) receptor ligands. CSF1-related pathways are required for macrophage proliferation, differentiation and even its own survival. Bone morphogenic protein 4 (BMP4) is also commonly applied during the first days of iPSC differentiation. BMP proteins are known to inhibit neurogenesis and induce neural stem cell (NSCs) glial differentiation in the adult CNS, particularly in subventricular zone, that results in the reduction of the stem cell pool [162,163].



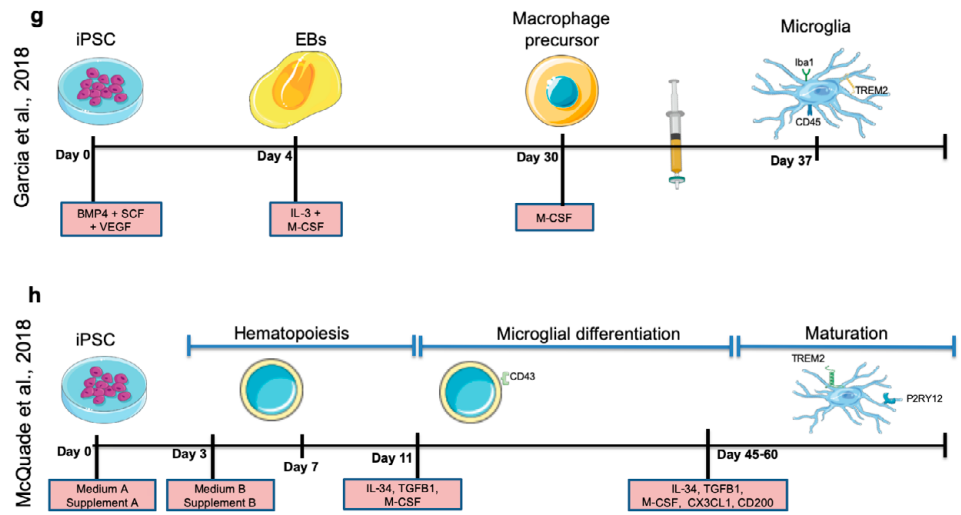


Figure 2. Outline of protocols to generate microglia from human iPSC. hiPSC are differentiated to embryonic macrophage precursor, microglia precursors cells by addition of specific growth factors and/or via embryoid bodies until they reach the maturation stage. Timelines are depicted in days.

BMP4 proteins belong to transforming growth factor β (TGF-β) superfamily, and beside their function in bone formation, they regulate proliferation and differentiation, cell-fate determination, and apoptosis. Some protocols employ embryonic bodies (EB) as an early step in the microglial generation and CSF1/ IL-34 [154] or IL-3/M-CSF [99, 101,102] as differentiation factors. To overcome potential variability in EB formation, and related batch-to-batch variability, Abud and colleagues directly differentiated iPSCs into hematopoietic progenitors by using FGF-2 and BMP4 [155]. In addition, they have used secreted proteins by neurons/ astrocytes/endothelial cells to mimic the natural microglial environment, by adding factors like TGF-β. All protocols validated differentiated microglial-like cells, by assessing their capacity to migrate, secrete cytokine/chemokine and also phagocyte, common functions mediated by human brain microglia.

Muffat et al. 2016, elaborated a protocol to create microglia from iPSC and ESC. First, human embryonic stem (hES) and induced pluripotent stem (iPS) cells have been cultivated in hES medium. Cystic and neutralized embryonic bodies (EB) were produced in a serum-free medium containing IL-34 and colony-stimulating factor 1 (CSF1). After 14 days, early yolk-sac myelogenesis markers became detectable, including VE-cadherin, c-kit CD41, CD235a, and, mainly PU.1, which is crucial for microglia maturation and viability [164]. Subsequently, EBs positive for yolk-sac markers were passed to polystyrene plates, where, after 30 days, semi-adherent cells exhibited a highly motile morphology and stained positive for markers such as PU.1, CD11B, and allograft inflammatory factor 1, which are well-defined microglia markers in several species [165]. The protocol expands over 56-60 days and the reported microglial yield was 1–8 × 10^6 pMGLs from 2 × 10^6 hPS.

To test the effectiveness of this protocol, Muffat et al., analyzed chemokines and cytokines under unstimulated and stimulated conditions with interferon γ (IFN-γ) and LPS. Before stimulation, the generated microglia, termed pluripotent stem cell-derived microglia-like cells (pMGLs) released various types of cytokines and chemokines, including IL-8, C-X-C motif chemokine ligand 1 (CXCL1) and C-C motif ligand 2 (CCL2). Under stimulation, the cells released these substances, but above baseline, in particular, CXCL10, CCL3 (or MIP1A), IL-6 and TNF-α - those last two were highly released and also expressed, as detected at transcriptional levels. Moreover, pMGLS appeared as a vastly ramified structure with slim end filaments, which resemble the phenotype of primary microglia. Transcriptomic data shown that pMGLs clustered with fetal microglia showing a unique signature. Besides, functional assays, as phagocytosis and migration were demonstrated in these differentiated pMGLs [154].

Abud et al. [155] reported that human induced microglial-like cells (iMGLs) could be generated after five weeks from iPSCs. First, iPSCs were differentiated into hematopoietic progenitors (iHSC) CD43+/CD235a+/CD41+. After ten days, iHSC CD43+ were cultivated in a serum-free differentiation medium containing CSF-1, IL-34, and transforming growth factor-β1 (TGFβ1). After more 14 days, the cells were positive for PU.1 and triggering receptor expressed on myeloid cells 2 (TREM2), and were grouped in CD45+/CX3CR1- and CD45+/CX3CR1+, which occurs equally *in vivo*. Following 35-38 days of differentiation, iMGL resemble human microglia and their gene profile started to diverge from macrophages and monocytes gene profile. They express several proteins such as MERTK, ITGBS, CX3CR1, TGFβR,1 and PROS1, which are microglial-enriched proteins, and purinergic receptors as P2RY12 and TREM2 [166]. Differentiated mature microglia was generated over a period of 38-40 days and the reported yield was 3–4 × 10^7 iMGLs from 1 × 10^6 hPSCs.

Culturing iPSC-derived microglia with factors that are normally produced and released by the surrounding healthy brain cells enabled them to exhibit a transcriptome profile similar to human fetal and adult microglia. Remarkably, this transcriptome profile is distinct from monocytes or blood dendritic cells. These factors include CX_3CL1, CD200 and TGFβ that highly mimic the surrounding microglial environment in the CNS, and provide a functional and relevant model to study microglial functions.

On transcriptomic level, iMGL clustered with human adult and fetal microglia [167,168]. Moreover, analyzing the cytokine/chemokine secretion and phagocytosis capacity the authors showed that iMGLs respond to their surface's receptors stimuli, resembling primary microglia activity. Their resemblance with primary microglia function was also demonstrated by the capacity of iMGLs to phagocyte human synaptosomes. Besides, iMGL responded well to ADP stimuli and were able to phagocyte fluorescently-labeled fibrillar Aβ and pHrodo-labeled brain-derived tau oligomers, indicating that this iMGL might represent a relevant model system to study AD pathology [169,170]. Moreover, iMGLs were co-cultured with rat hippocampal neurons, which increased the gene expression of neuroprotective function and decreased pro-inflammatory genes. Additionally, they included iMGLs in the brain cortex of mice and demonstrated the cells ability to engraft and survive into real CNS environment.

Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

Douvaras et al. 2017 [156] developed a protocol in which myeloid progenitors generated microglia-like cells. First, the human PS cells were grown in feeder-free media with bone morphogenetic-protein 4 (BMP4) for four days, to generate primitive hemangioblasts. Then, medium containing basic fibroblast growth factor (bFGF), stem cell factor (SCF), and vascular endothelial growth factor A ($VEGF_A$) was added for additional two days. During the next eight days (6-14), the factors in the medium were replaced by interleukin-3 (IL-3), thrombopoietin (TPO), SCF, macrophage colony-stimulating factor (M-CSF) and FMS-like tyrosine kinase 3 (FTL3). From day 14 to 25 medium was supplemented with M-CSF, FLT3, and granulocyte macrophage colony-stimulating factor (GM-CSF). Microglia progenitors CD14+/CX3CR1+ were isolated by FACS and were placed in contact with medium with IL-34 and GM-CSF for one to two weeks to generate microglia. Differentiated mature microglia was generated over a period of 35-60 days and the reported yield was 2–3 × 10^6 iPSC-MG from 1 × 10^6 hPSCs. To evaluate iPSC-microglia, Douvaras et al., 2017 clustered iPSC-microglia with human fetal microglia (hMG), and they confirmed the expression of six-genes specific to human microglia [167,171]. In addition to the gene characterization, the cytokine/chemokine profile and the phagocytosis assay validated the functionality of mature microglia. P2RY12, a gene that encodes a G1 protein was capable of inducing intracellular Ca^{2+} transients in response to ADP in iPSC-microglia resembling the activity of primary microglia [172,173].

Haenseler et al. 2017 [157] described a protocol based on the study of van Wilgenburg et al. 2013 [174] generating microglia starting from embryonic-like myeloblastosis (MYB)-independent macrophage precursors. Following one month of differentiation, macrophage precursors were harvested in the supernatant, and the cells were collected and co-cultured with iPSC-derived cortical neurons in a medium enriched with IL-34 and M-CSF. Two weeks following co-culturing, macrophage precursors had a similar phenotype as primary microglia, with ramified branches (co-pMG). Differentiated mature microglia was generated over a period of 30 days and the reported yield was 1–4 × 10^7 pMacpre from 1 × 10^6 hPS. Transcriptomic analysis corroborated than iPSC microglia was clustered with fetal microglia and showed 6 specific genes for microglia; also, protein expression for Iba-1, P2YR12, TMEM119, and MERTK. Besides, gene ontology assays demonstrated a downregulation of genes involved in viral, bacterial and yeast recognition response and upregulation of genes responsible for survival as differentiation, chemotaxis, regulation cell-cell adhesion, and metal ion response was evidenced [175]. iPSC microglia were able to respond to LPS/ IFN-γ stimulation and to promote phagocytosis.

Pandya et al., 2017 [158] developed a protocol based on a co-culture with astrocytes where iPSC were cultured in medium with VEGF, BMP4, SCF, and ActivinA for four days. Afterwards, new growth factors were added to the medium (Flt3, IL-3, IL-6, F-CSF) for more ten days. On day 15, cells expressed myeloid progenitors' markers: CD34 and CD43. These cells were co-cultured with human astrocytes in a medium containing GM-CSF, M-CSF, and IL-3 for additional two weeks. At this stage, the differentiated cells were positive for microglial markers, such as CD11b and Iba1. The protocol expands over 30-60 days and the reported microglial yield was 1–3 × 10^6 iPS-MG from 1 × 10^6 hPS. Gene expression signature of iPS-MG was evaluated and it displayed clusters with human fetal microglia and also with

dendritic cells and macrophages, resembling their myeloid lineage. Phagocytic activity and ROS production were assessed using pHrodo *E. coli* Bio Particles and phorbol myristate acetate (PMA), respectively and demonstrated the capacity of iPS-MG to respond to various stimuli, including LPS and TNF-α cytokine.

Brownjohn *et al.*, 2018 and Garcia et al., 2018 developed similar protocols to generate microglia from iPSC trying to understand TREM2 mutations in neurodegenerative diseases [159,160]. They divided their protocol into two phases: the differentiation of iPSC in primitive macrophages precursor (PMP) and then in iPSC-microglia. First, embryoid bodies were developed in ultra-low attachment plates with medium containing BMP-4, SCF, and VEGF-121 for four days. EBs were exposed to IL-3 and M-CSF for additional 3-4 weeks. After this, PMP were cultured in enriched medium with GM-CSF and IL-34 for 6-10 days in Brownjohn protocol. In Garcia protocol a 40-micron filter was used to separate cells and the induction of cell maturation was performed in medium containing only M-CSF. Functional studies such as phagocytic assays, injury responses, and physiological responses complemented transcriptomic studies and probed the resembling to primary microglia.

One of the most recent protocol was presented by McQuade et al., 2018. After seeding, cells were exposed to medium A (Supplement A), after three days, the medium was changed to medium B (Supplement B). On day 10-12, non-adherent cells were positive for CD43 and termed Hematopoietic progenitor cells (HPC). Their medium was supplemented with IL-34, TGFβ1, and M-CSF and after additional 4-6 weeks medium was changed with new growth factors (IL-34, TGFβ1, M-CSF, CD200, and CX3CL1) to further ensure the microglial maturation and cellular homeostasis. To evaluate the functional activity of iPS-microglia, phagocytic activity was demonstrated by exposure of microglia to different stimuli. Transcriptomic analysis of generated iPS-microglia presented an exclusive profile with genes resembling primary microglia and distinct from hematopoietic progenitor cells, monocytes, and dendritic cells.

The first protocols of microglial differentiation showed maturation and fully functional microglia following 60-75 days in culture. More recent protocols demonstrated that microglia can be differentiated from iPSCs in only 24 days according to Konttinen et al., 2019 [176]. The protocol they developed involved specific oxygen concentrations in the first stages of differentiation. 48 hours after seeding, mesodermal cells were treated with bFGF, VEGF, and insulin to induce hemogenic differentiation. Then, cells were subjected to MCSF1 and IL-34 to induce microglial differentiation. To mature the cells, they were cultured on poly-D-lysine (PDL)-coated plates until D24, when they expressed IBA1. On their study of iPSC-derived microglia, APOE4 mutation exhibited profound impact on fundamental aspects of microglial function such as phagocytosis, migration and metabolism, supporting the hypothesis of impairment of microglial function by APOE4 [177]. Interestingly, APPswe, and Psen1 mutations had minor effects. Moreover, the authors acknowledge that these microglia, may represent relatively young microglia based on the expression of P2RY12, a marker of mature microglia.

Despite encouraging results, caution must be taken to interpret these data, as microglia in this study were differentiated in a fetal bovine serum (FBS)-containing media. FBS is not a well-defined supplement and may promote microglial priming, which can mask differences between groups. All in all, this recent study proposed a short differentiation protocol for microglia.

Transcriptomic analysis of microglia obtained from differentiation protocols.

Efforts to standardize protocols to differentiate microglia from patient-derived iPSCs have led to identification of transcriptomic signatures that support the use of these microglia-like cells as an alternative to human primary microglia. For instance, the transcriptomic clustering approach has been utilized to highlight similarities and differences between microglia-like cells, other CNS-cells, myeloid cells, iPSCs, fetal and adult microglia [154,155,157,160,161,176].

Regarding their similarity to human microglia, Muffat et al found that their pluripotent stem cell-derived microglia-like cells (pMGLs) did not differ in any of the canonical myeloid ontology terms with human fetal microglia [154]. As the first study to propose the use of iPSC-derived cells as surrogates for human microglia, it paved the way for further transcriptomic and functional characterization of pMGLs. However, this study did not assess the similarity to adult microglia. Later, Abud and colleages compared their human microglial-like cells (iMGLs) with adult microglia finding important similarities, especially in the expression of CD11b, ITGB2, CSF1R, CD45, IBA1, LGMN[155].

By correlation and principal component analysis (PCA) of whole transcriptome, Abud et al. have shown that their iMGLs do not cluster with other myeloid cells, such as $CD14^{+}$/ $CD16^{+/-}$ monocytes and blood dendritic cells (DCs)[155]. Additionally, García et al., Douvaras et al. and Haenseler et al. focused on genes proposed by Butovsky et al., 2014 to be preferentially expressed in microglia, such as TREM2, C1QA, TMEM119, GPR34, PROS1 but not in monocytes[156,157,160,178]. These genes showed to be expressed in microglia but not in peripheral blood monocytes and primary macrophages.

More recent protocols employed in studies by McQuade and colleagues focused in the comparison of the so-called iPS-microglia 2.0 with the previously published iPSC-microglia from Douvaras and colleagues[161]. This study concluded that iPSC-microglia 2.0, which result from a less complex protocol, are virtually identical to iPSC-microglia regarding to their transcriptomic profile. Similarly, they study of Konttinen and colleagues compared their iMGL transcriptome data with Abud's, concluding that they clustered with human microglia and published iMGLs [176]. All in all, transcriptomic analysis of iPSC-derived microglia-like cells is a valuable tool to understanding function and evaluate expected responses to overcome the culprits and technical difficulties of primary microglia harvesting and culture.

Microglia in brain organoids

Limitations of 2-dimensional (2D) culture systems to replicate and evaluate the human brain pathologies, have led to the generation of 3D culture systems or brain organoids/spheroids/assembloids from human iPSCs by direct replicating the neurological development.

At the moment, there are a variety of strategies available to develop brain organoids/spheroids/assembloids as for other types of tissues or organs, such as retina, intestine, thyroid, liver, inner ear, pituitary gland, and kidney [179–181]. Cerebral assembloids are stem-cell derived models in a three dimensional *in vitro* culture systems that aim to recapitulate the developmental processes and structural brain organization of the developing or adult human brain [3]. Current 3D assembloids are able to accurately summarize defects in early brain development as nicely demonstrated for microcephaly by Lancaster et al. 2013 [182] and for Zika virus infection by Qian et al. 2016 [183].

To create a 3D assembloid similar to the human brain is complex, as it must contain different neural populations, astrocytes, oligodendrocytes, microglia and the blood brain barrier cell population. Since microglia has another embryonic origin than neuronal or astrocytic cells, the generation of assembloids that are spontaneously populated with microglial cells has been proven to be a difficult task [182,184]. However, recent studies proposed novel strategies to create an assembloid with cells of all germinal layers, including microglia. This combination enables a better understanding of a healthy brain and also the pathophysiology of neurodegenerative disorders, since interactions between microglia and macroglia/neurons are crucial during brain development, and aging [185,186].

Brain assembloids have a certain degree of resemblances to *in vivo* conditions, although some features and cell interactions still cannot be reproduced. Interactions between microglia and brain-blood barrier (BBB) can be relevant in the context of neuroinflammation, considering that microglia could be activated by changes in the BBB permeability, that is often compromised in age-related neurodegenerative diseases [187–189]. Further, Erny and colleagues described the importance of host microbiota to microglia maturation, morphology, and function [190]. More studies are necessary to understand the relationship between microglia and neural populations in neurodegenerative diseases. Moreover, to model neuroimunological interactions in human brain and investigate the consequence of these interactions on brain pathology, researchers combined brain neuronal assembloids with immune cells, such as microglia-like cells [155] [191].

Hitherto, only a limited number of studies have showed the interaction of neurons and microglia in a 3D setting, by incorporation of differentiated microglia in brain organoids/assembloids, as illustrated in Figure 3. Muffat and colleagues added the differentiated pMGLs in a 3D brain structure of a three months old spheroid. NPCs were used to generate the 3D

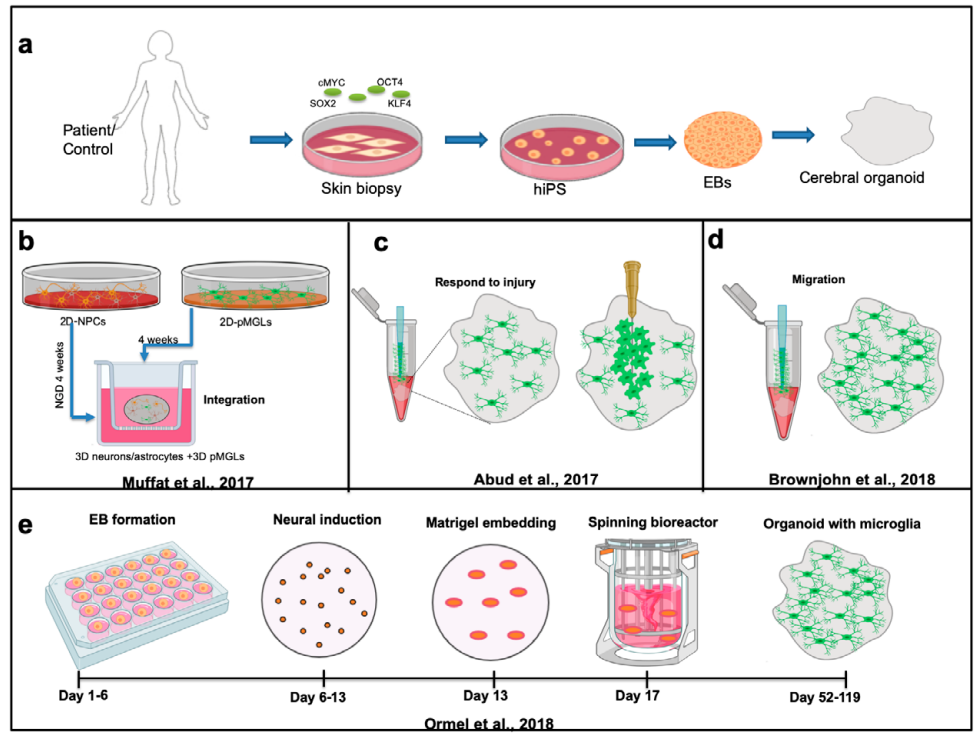


Figure 3. A scheme for iPSC modeling and microglia in organoids. (**a)** Generation of organoids using patient-derived iPSCs mainly includes the following steps. First, iPSCs are generated from patients by reprogramming somatic cells using 4 Yamanaka factors, OCT4, SOX2, KLF4, and MYC. Human-induced pluripotent stem cells will create embryonic bodies (EBs) and consequently, cerebral organoids with different growth factors, mediums, and reagents. (**b)** Graphic representation of direct re-aggregation of microglia, astrocytes and neurons in spheroids or 3D stacks in transwells, according to Muffat et al., 2016 [154]. (**c)** Abud et al.,2017 [155] showed how ameboid microglia was localized close to damaged area (pierce with 25G needle) in cerebral organoids. **(d)** Microglia migrate into preformed cortical organoids and assume a pronounced ramified morphology, which is demonstrated by Brownjohn et al.,2018 [159]. **(e)** Ormel et al., 2018 [192] developed a protocol where the organoid by itself shows microglia after two months.

brain-like structure which was populated by neurons, oligodendrocytes, and astroglia [154]. GFP-labeled pMGLs microglia exhibited a rounded morphology when cultured on plastic in a 2D shape; however, in the 3D environment, microglia displayed highly ramified branches. In a similar fashion, Abud, Brownjohn and colleagues added iMGLs to organoids and showed that microglia migrated from the surface deeply into organoid and, upon integration, adopted a highly ramified morphology, surviving in those environments for several weeks [155,159]. Besides, following an induced injury with a needle, iMGLs migrated and clustered near the injury needle sit, acquiring a more amoeboid morphology, resembling ‘‘activated’’ microglia observed in response to injury or neurodegeneration [155].

Recently, Ormel and colleagues [192] showed that microglia can innately develop within a cerebral organoid (oMG) model and exhibit their 3D characteristic ramified morphology. The

oMG was IBA-1 positive following 2 months of culturing in the cerebral organoid. Extensively transcriptomic assays were assessed comparing oMG with adult and fetal primary human microglia, 2D iPSC microglia, iPSC, and fibroblasts. Interestingly, oMG clustered with adult primary microglia, whereas 2D iPSC microglia clustered with fetal primary microglia [192]. Besides, stimulation with pro- or anti-inflammatory triggers - LPS and dexamethasone, respectively – led to an increased cytokine release (IL-6 and IL-1β) in oMG and a comparable response for dexamethasone (CD163 and MRC1) with adult primary microglia.

To understand how microglia affect βA clearance in AD, Lin and colleagues cultured human differentiated microglia with two-month-old familial AD-derived forebrain organoids that have an increased expression of amyloid precursor proteins (APP) [191]. Microglia was generated from iPSC derived from AD patients carrying either a low-risk gene variant (APOE3) or a high-risk gene variant (APOE4). One month of co-culture rendered comparable numbers of microglia integrated into organoids regardless of APOE genotype. However, the morphology and function of microglia-like cells derived from a high susceptibility background, the APOE4 variant was different than the APOE3 microglia-like cells, with longer processes and reduced capacity to phagocyte βA. As a result, organoids populated by APOE3 microglia-like cells contained fewer βA aggregates compared to organoids with APOE4 microglia. Morphological alterations in APOE4 microglia correlated well with the capacity of βA uptake, that potentially restricts the ability of microglia to clear extracellular βA plaques from AD brains [191].

Most of microglial derivations lack specificity regarding region-specific microenvironment. For instance, forebrain microglia depend on IL-34 for maintenance, while cerebellar does not. Work from Song and colleagues was the first to address this issue by generating dorsal and ventral brain organoids and co-culturing them with microglia[193]. Interestingly, dorsal organoids showed higher anti-inflammatory cytokine secretion, while ventral organoids exhibited higher TNF-α expression. Transcriptomic analysis exhibited microglia-specific genes that were differentially expressed in both groups. Regarding disease modeling, findings from this study add a layer of complexity to more accurately resembling diseased region-specific microenvironments.

A more sophisticated cell model system was used by Park and colleagues to study brain cell interactions in a microfluidic-based system. The microfluidic chambers contained neurons and astrocytes differentiated from NPCs cultured together with a human immortalized SV40 microglia-like cell line. The culturing was realized in a microfluidic-based system where neuroimmunologic interactions related to AD pathology could be easily modeled and tested. Neuronal cells and astrocytes were differentiated from ReNcell VM cells (immortalized hiPSC-derived hNPCs) expressing multiple familial AD mutations, including APP mutations (mAPP). Microglial morphology and activity were altered in the presence of mAPP neurons/

astrocytes, with microglia migrating faster towards βA aggregates and causing cell death to mAPP neurons/astrocytes. Using this model system, Park and colleagues tested different pathways targeting microglia-neuron interaction, including anti-CCL2 neutralizing antibodies or knockdown of TLR4 in microglia. These strategies reduced microglial migration and also neuronal toxicity of mAPP neurons/astrocytes, providing insights into the cytokine signaling pathways activated and potentially druggable in AD [194].

Taken together these studies, underline the importance of microglia in cerebral organoids as a tool to study the effects of cell interactions on CNS during development, maturation, inflammation, and neurodegenerative diseases.

Conclusions

Microglia are the resident immune surveillance cells within the CNS and are involved in a plethora of physiological as well as pathophysiological functions. Microglial role in neurodegenerative diseases, such as PD, AD, ALS is complex and there is an urgent need to understand better the pathways that regulate their proinflammatory response to injury. Recently, microglia *in vitro* models have been established with respective advantages and disadvantages as summarized previously. Lately, ESC and iPSC-derived 2D and 3D models, in combination with exposure to CNS microenvironmental cues, form a strong basis to pursue studies of microglial biology in health and disease. Besides, it brings the opportunity to study stem cell-derived 3D human brain organoids/assembloids where one can recapitulate features of the human brain with greater complexity than the classical Petri-dish 2D models. Considering that neuroinflammation is involved in neurological diseases, generation of a brain organoid/assembloid with all neural population will form an environment where human microglia interacts with other brain populations offering a relevant model to study brain function and pathologies. A better comprehension of inflammatory pathways and novel reliable, easily reproducible and relevant human stem cell-based models will represent an important step in our understanding of the pathogenesis of neurodegenerative disease, hence finding efficient therapies.

References

[1] M.-È. Tremblay, C. Lecours, L. Samson, V. Sánchez-Zafra, A. Sierra, From the Cajal alumni Achúcarro and Río-Hortega to the rediscovery of never-resting microglia, Front. Neuroanat. 9 (2015) 1–10. doi:10.3389/fnana.2015.00045.

[2] M. Mittelbronn, K. Dietz, H.J. Schluesener, R. Meyermann, Local distribution of microglia in the normal adult human central nervous system differs by up to one order of magnitude, Acta Neuropathol. 101 (2001) 249–255. doi:10.1007/s004010000284.

[3] C. Schulz, E. Gomez Perdiguero, L. Chorro, H. Szabo-Rogers, N. Cagnard, K. Kierdorf, M. Prinz, B. Wu, S.E.W. Jacobsen, J.W. Pollard, J. Frampton, K.J. Liu, F. Geissmann, A lineage of myeloid cells independent of Myb and hematopoietic stem cells., Science. 336 (2012) 86–90. doi:10.1126/science.1219179.

[4] F. Ginhoux, M. Greter, M. Leboeuf, S. Nandi, P. See, S. Gokhan, M.F. Mehler, S.J. Conway, L.G. Ng, E.R. Stanley, I.M. Samokhvalov, M. Merad, Fate mapping analysis reveals that adult microglia derive from primitive macrophages, Science. 330 (2010) 841–845. doi:10.1126/science.1194637.

[5] K. Kierdorf, D. Erny, T. Goldmann, V. Sander, C. Schulz, E.G. Perdiguero, P. Wieghofer, A. Heinrich, P. Riemke, C. Hölscher, others, Microglia emerge from erythromyeloid precursors via Pu.1 and Irf8-dependent pathways, Nat. Neurosci. 16 (2013) 273–283. doi:doi:10.1038/nn.3318.

[6] M. Prinz, J. Priller, Microglia and brain macrophages in the molecular age: From origin to neuropsychiatric disease, Nat. Rev. Neurosci. 15 (2014) 300–312. doi:10.1038/nrn3722.

[7] K. Askew, K. Li, A. Olmos-Alonso, F. Garcia-Moreno, Y. Liang, P. Richardson, T. Tipton, M.A. Chapman, K. Riecken, S. Beccari, A. Sierra, Z. Molnár, M.S. Cragg, O. Garaschuk, V.H. Perry, D. Gomez-Nicola, Coupled Proliferation and Apoptosis Maintain the Rapid Turnover of Microglia in the Adult Brain, Cell Rep. 18 (2017) 391–405. doi:10.1016/j.celrep.2016.12.041.

[8] S. Jäkel, L. Dimou, Glial Cells and Their Function in the Adult Brain: A Journey through the History of Their Ablation, Front. Cell. Neurosci. 11 (2017) 1–17. doi:10.3389/fncel.2017.00024.

[9] V. Mondelli, A.C. Vernon, F. Turkheimer, P. Dazzan, C.M. Pariante, Brain microglia in psychiatric disorders, The Lancet Psychiatry. 4 (2017) 563–572. doi:10.1016/S2215-0366(17)30101-3.

[10] B. Rock, G. Gekker, S. Hu, W. Sheng, M. Cheeran, J. Lokensgard, P. Peterson, Role of Microglia in Central Nervous System Infections, Clin. Microbiol. Rev. 17 (2004) 942–964. doi:10.1128/CMR.17.4.942–964.2004.

[11] K.M. Lenz, L.H. Nelson, Microglia and Beyond: Innate Immune Cells As Regulators of Brain Development and Behavioral Function, Front. Immunol. 9 (2018) 698. doi:10.3389/fimmu.2018.00698.

[12] L. Sevenich, Brain-Resident Microglia and Blood-Borne Macrophages Orchestrate Central Nervous System Inflammation in Neurodegenerative Disorders and Brain Cancer, Front. Immunol. 9 (2018) 697. doi:10.3389/fimmu.2018.00697.

[13] J.Y. Bertrand, A. Jalil, M. Klaine, S. Jung, A. Cumano, I. Godin, Three pathways to mature macrophages in the early mouse yolk sac, Blood. (2005). doi:10.1182/blood-2005-02-0461.

[14] G. Hoeffel, Y. Wang, M. Greter, P. See, P. Teo, B. Malleret, M. Leboeuf, D. Low, G. Oller, F. Almeida, S.H.Y. Choy, M. Grisotto, L. Renia, S.J. Conway, E.R. Stanley, J.K.Y. Chan, L.G. Ng, I.M. Samokhvalov, M. Merad, F. Ginhoux, Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac–derived macrophages, J. Exp. Med. 209 (2012) 1167 LP – 1181. doi:10.1084/jem.20120340.

[15] C.T. Lux, M. Yoshimoto, K. McGrath, S.J. Conway, J. Palis, M.C. Yoder, All primitive and definitive hematopoietic progenitor cells emerging before E10 in the mouse embryo are products of the yolk sac, Blood. 111 (2008) 3435–3438. doi:10.1182/blood-2007-08-107086.

[16] J. Palis, S. Robertson, M. Kennedy, C. Wall, G. Keller, Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse, Development. (1999).

[17] G. Hoeffel, J. Chen, Y. Lavin, D. Low, F.F. Almeida, P. See, A.E. Beaudin, J. Lum, I. Low, E.C. Forsberg, M. Poidinger, F. Zolezzi, A. Larbi, L.G. Ng, J.K.Y. Chan, M. Greter, B. Becher, I.M. Samokhvalov, M. Merad, F. Ginhoux, C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages, Immunity. 42 (2015) 665–678. doi:10.1016/j.immuni.2015.03.011.

[18] K. Kierdorf, D. Erny, T. Goldmann, V. Sander, C. Schulz, E.G. Perdiguero, P. Wieghofer, A. Heinrich, P. Riemke, C. Hölscher, D.N. Müller, B. Luckow, T. Brocker, K. Debowski, G. Fritz, G. Opdenakker, A. Diefenbach, K. Biber, M. Heikenwalder, F. Geissmann, F. Rosenbauer, M. Prinz, Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways, Nat. Neurosci. 16 (2013) 273.

[19] I.M. Chiu, E.T.A. Morimoto, H. Goodarzi, J.T. Liao, S. O'Keeffe, H.P. Phatnani, M. Muratet, M.C. Carroll, S. Levy, S. Tavazoie, R.M. Myers, T. Maniatis, A neurodegeneration-specific gene-expression signature of acutely isolated microglia from an amyotrophic lateral sclerosis mouse model, Cell Rep. 4 (2013) 385–401. doi:10.1016/j.celrep.2013.06.018.

[20] S.E. Hickman, N.D. Kingery, T.K. Ohsumi, M.L. Borowsky, L. Wang, T.K. Means, J. El Khoury, The microglial sensome revealed by direct RNA sequencing, Nat. Neurosci. 16 (2013) 1896–1905. doi:10.1038/nn.3554.

[21] O. Butovsky, M.P. Jedrychowski, C.S. Moore, R. Cialic, A.J. Lanser, G. Gabriely, T. Koeglsperger, B. Dake, P.M. Wu, C.E. Doykan, Z. Fanek, L. Liu, Z. Chen, J.D. Rothstein, R.M. Ransohoff, S.P. Gygi, J.P. Antel, H.L. Weiner, Identification of a unique TGF-β-dependent molecular and functional signature in microglia, Nat. Neurosci. (2014). doi:10.1038/nn.3599.

[22] D. Gosselin, V.M. Link, C.E. Romanoski, G.J. Fonseca, D.Z. Eichenfield, N.J. Spann, J.D. Stender, H.B. Chun, H. Garner, F. Geissmann, C.K. Glass, Environment drives selection and function of enhancers controlling tissue-specific macrophage identities, Cell. 159 (2014) 1327–1340. doi:10.1016/j.cell.2014.11.023.

[23] Y. Lavin, D. Winter, R. Blecher-Gonen, E. David, H. Keren-Shaul, M. Merad, S. Jung, I. Amit, Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment, Cell. 159 (2014) 1312–1326. doi:10.1016/j.cell.2014.11.018.

[24] H.C. Bauer, H. Bauer, A. Lametschwandtner, A. Amberger, P. Ruiz, M. Steiner, Neovascularization and the appearance of morphological characteristics of the blood-brain barrier in the embryonic mouse central nervous system, Dev. Brain Res. (1993). doi:10.1016/0165-3806(93)90031-5.

[25] O. Matcovitch-Natan, D.R. Winter, A. Giladi, S. Vargas Aguilar, A. Spinrad, S. Sarrazin, H. Ben-Yehuda, E. David, F. Zelada González, P. Perrin, H. Keren-Shaul, M. Gury, D. Lara-Astaiso, C.A. Thaiss, M. Cohen, K. Bahar Halpern, K. Baruch, A. Deczkowska, E. Lorenzo-Vivas, S. Itzkovitz, E. Elinav, M.H. Sieweke, M. Schwartz, I. Amit, Microglia development follows a stepwise program to regulate brain homeostasis, Science (80-.). 353 (2016) aad8670. doi:10.1126/science.aad8670.

[26] A.M. Smith, M. Dragunow, The human side of microglia, Trends Neurosci. (2014). doi:10.1016/j.tins.2013.12.001.

[27] A. Monier, P. Evrard, P. Gressens, C. Verney, Distribution and differentiation of microglia in the human encephalon during the first two trimesters of gestation, J. Comp. Neurol. (2006). doi:10.1002/cne.21123.

[28] E.E. Spangenberg, R.J. Lee, A.R. Najafi, R.A. Rice, M.R.P. Elmore, M. Blurton-Jones, B.L. West, K.N. Green, Eliminating microglia in Alzheimer's mice prevents neuronal loss without modulating amyloid-β pathology, Brain. 139 (2016) 1265–1281. doi:10.1093/brain/aww016.

[29] M. Li, Z. Li, H. Ren, W.-N. Jin, K. Wood, Q. Liu, K.N. Sheth, F.-D. Shi, Colony stimulating factor 1 receptor inhibition eliminates microglia and attenuates brain injury after intracerebral hemorrhage, J. Cereb. Blood Flow Metab. 37 (2017) 2383–2395. doi:10.1177/0271678X16666551.

[30] G. Szalay, B. Martinecz, N. Lénárt, Z. Környei, B. Orsolits, L. Judák, E. Császár, R. Fekete, B.L. West, G. Katona, B. Rózsa, Á. Dénes, Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke, Nat. Commun. 7 (2016) 11499. doi:10.1038/ncomms11499.

[31] M.M. Acharya, K.N. Green, B.D. Allen, A.R. Najafi, A. Syage, H. Minasyan, M.T. Le, T. Kawashita, E. Giedzinski, V.K. Parihar, B.L. West, J.E. Baulch, C.L. Limoli, Elimination of microglia improves cognitive function following cranial irradiation, Sci. Rep. 6 (2016) 31545. doi:10.1038/srep31545.

[32] M.R.P. Elmore, R.J. Lee, B.L. West, K.N. Green, Characterizing newly repopulated microglia in the adult mouse: impacts on animal behavior, cell morphology, and neuroinflammation, PLoS One. 10 (2015) e0122912–e0122912. doi:10.1371/journal.pone.0122912.

[33] S.A. Chalmers, J. Wen, J. Shum, J. Doerner, L. Herlitz, C. Putterman, CSF-1R inhibition attenuates renal and neuropsychiatric disease in murine lupus, Clin. Immunol. 185 (2017) 100–108. doi:10.1016/j.clim.2016.08.019.

[34] L. Torres, J. Danver, K. Ji, J.T. Miyauchi, D. Chen, M.E. Anderson, B.L. West, J.K. Robinson, S.E. Tsirka, Dynamic microglial modulation of spatial learning and social behavior, Brain. Behav. Immun. 55 (2016) 6–16. doi:10.1016/j.bbi.2015.09.001.

[35] M.R.P. Elmore, A.R. Najafi, M.A. Koike, N.N. Dagher, E.E. Spangenberg, R.A. Rice, M. Kitazawa, B. Matusow, H. Nguyen, B.L. West, K.N. Green, Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain, Neuron. (2014). doi:10.1016/j.neuron.2014.02.040.

[36] L.H. Nelson, K.M. Lenz, Microglia depletion in early life programs persistent changes in social, mood-related, and locomotor behavior in male and female rats, Behav. Brain Res. 316 (2017) 279–293. doi:10.1016/j.bbr.2016.09.006.

[37] J.W. VanRyzin, S.J. Yu, M. Perez-Pouchoulen, M.M.

McCarthy, Temporary Depletion of Microglia during the Early Postnatal Period Induces Lasting Sex-Dependent and Sex-Independent Effects on Behavior in Rats, ENeuro. 3 (2016) ENEURO.0297-16.2016. doi:10.1523/ENEURO.0297-16.2016.

[38] A. Waisman, F. Ginhoux, M. Greter, J. Bruttger, Homeostasis of Microglia in the Adult Brain: Review of Novel Microglia Depletion Systems, Trends Immunol. (2015). doi:10.1016/j.it.2015.08.005.

[39] G. Gowing, L. Vallières, J.P. Julien, Mouse model for ablation of proliferating microglia in acute CNS injuries, Glia. (2006). doi:10.1002/glia.20288.

[40] R.E. Bennett, D.L. Brody, Acute reduction of microglia does not alter axonal injury in a mouse model of repetitive concussive traumatic brain injury, J. Neurotrauma. (2014). doi:10.1089/neu.2013.3320.

[41] N.H. Varvel, S.A. Grathwohl, F. Baumann, C. Liebig, A. Bosch, B. Brawek, D.R. Thal, I.F. Charo, F.L. Heppner, A. Aguzzi, O. Garaschuk, R.M. Ransohoff, M. Jucker, Microglial repopulation model reveals a robust homeostatic process for replacing CNS myeloid cells, Proc. Natl. Acad. Sci. U. S. A. (2012). doi:10.1073/pnas.1210150109.

[42] C.N. Parkhurst, G. Yang, I. Ninan, J.N. Savas, J.R. Yates, J.J. Lafaille, B.L. Hempstead, D.R. Littman, W.B. Gan, Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor, Cell. (2013). doi:10.1016/j.cell.2013.11.030.

[43] J. Bruttger, K. Karram, S. Wörtge, T. Regen, F. Marini, N. Hoppmann, M. Klein, T. Blank, S. Yona, Y. Wolf, M. Mack, E. Pinteaux, W. Müller, F. Zipp, H. Binder, T. Bopp, M. Prinz, S. Jung, A. Waisman, Genetic Cell Ablation Reveals Clusters of Local Self-Renewing Microglia in the Mammalian Central Nervous System, Immunity. (2015). doi:10.1016/j.immuni.2015.06.012.

[44] P. Wieghofer, M. Prinz, Genetic manipulation of microglia during brain development and disease, Biochim. Biophys. Acta - Mol. Basis Dis. (2016). doi:10.1016/j.bbadis.2015.09.019.

[45] P. Squarzoni, M.S. Thion, S. Garel, Neuronal and microglial regulators of cortical wiring: Usual and novel guideposts, Front. Neurosci. 9 (2015) 1–16. doi:10.3389/fnins.2015.00248.

[46] L. Fourgeaud, P.G. Través, Y. Tufail, H. Leal-bailey, D. Erin, P.G. Burrola, P. Callaway, A. Zagórska, C. V Rothlin, TAM receptors regulate multiple features of microglial physiology, 532 (2016) 240–244. doi:10.1038/nature17630.TAM.

[47] H. Kettenmann, F. Kirchhoff, A. Verkhratsky, Microglia: New Roles for the Synaptic Stripper, Neuron. 77 (2013) 10–18. doi:10.1016/j.neuron.2012.12.023.

[48] M.-E. Tremblay, B. Stevens, A. Sierra, H. Wake, A. Bessis, A. Nimmerjahn, The Role of Microglia in the Healthy Brain, J. Neurosci. 31 (2011) 16064–16069. doi:10.1523/jneurosci.4158-11.2011.

[49] A. Aguzzi, B.A. Barres, M.L. Bennett, Microglia: Scapegoat, Saboteur, or Something Else ?, Science (80-.). 339 (2013) 156–162. doi:10.1126/science.1227901.

[50] A. Vezzani, B. Viviani, Neuromodulatory properties of inflammatory cytokines and their impact on neuronal excitability, Neuropharmacology. 96 (2015) 70–82. doi:10.1016/j.neuropharm.2014.10.027.

[51]A. Masuch, C.H. Shieh, N. van Rooijen, D. van Calker, K. Biber, Mechanism of microglia neuroprotection: Involvement of P2X7, TNFα, and valproic acid, Glia. 64 (2015) 76–89. doi:10.1002/glia.22904.

[52] M. Lee, C. Schwab, P.L. Mcgeer, Astrocytes are GABAergic cells that modulate microglial activity, Glia. 59 (2011) 152–165. doi:10.1002/glia.21087.

[53] J. V. Welser-Alves, S.J. Crocker, R. Milner, A dual role for microglia in promoting tissue inhibitor of metalloproteinase (TIMP) expression in glial cells in response to neuroinflammatory stimuli, J. Neuroinflammation. 8 (2011) 61. doi:10.1186/1742-2094-8-61.

[54] M. Colonna, O. Butovsky, Microglia Function in the Central Nervous System During Health and Neurodegeneration, Annu. Rev. Immunol. 35 (2017) 441–68. doi:10.1146/annurev-immunol-051116-052358.

[55] A. Nimmerjahn, F. Kirchhoff, F. Helmchen, Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo, Science (80-.). 308 (2005) 1314–1318. doi:10.1126/science.1110647.

[56] R. Timmerman, S.M. Burm, J.J. Bajramovic, An Overview of in vitro Methods to Study Microglia, Front. Cell. Neurosci. 12 (2018) 1–12. doi:10.3389/fncel.2018.00242.

[57] H.O. Kalkman, D. Feuerbach, Antidepressant therapies inhibit inflammation and microglial M1-polarization, Pharmacol. Ther. 163 (2016) 82–93. doi:10.1016/j.pharmthera.2016.04.001.

[58] H.J. Park, S.H. Oh, H.N. Kim, Y.J. Jung, P.H. Lee, Mesenchymal stem cells enhance α-synuclein clearance via M2 microglia polarization in experimental and human parkinsonian disorder, Acta Neuropathol. 132 (2016) 685–701. doi:10.1007/s00401-016-1605-6.

[59] L.A.N. Peferoen, D.Y.S. Vogel, K. Ummenthum, M. Breur, P.D.A.M. Heijnen, W.H. Gerritsen, R.M.B. Peferoen-Baert, P. Van Der Valk, C.D. Dijkstra, S. Amor, Activation status of human microglia is dependent on lesion formation stage and remyelination in multiple sclerosis, J. Neuropathol. Exp. Neurol. 74 (2015) 48–63. doi:10.1097/NEN.0000000000000149.

[60] M. Olah, K. Biber, J. Vinet, H. W.G.M. Boddeke, Microglia Phenotype Diversity, CNS Neurol. Disord. - Drug Targets. 10 (2011) 108–118. doi:10.2174/187152711794488575.

[61] L.C. Walker, M. Jucker, Neurodegenerative Diseases: Expanding the Prion Concept, Annu. Rev. Neurosci. 38 (2015) 87–103. doi:10.1146/annurev-neuro-071714-033828.

[62] D.M. Skovronsky, V.M.-Y. Lee, J.Q. Trojanowski, NEURODEGENERATIVE DISEASES: New Concepts of Pathogenesis and Their Therapeutic Implications, Annu. Rev. Pathol. Mech. Dis. 1 (2006) 151–170. doi:10.1146/annurev.pathol.1.110304.100113.

[63] C.K. Glass, K. Saijo, B. Winner, M.C. Marchetto, H. Gage, Mechanisms Underlying Inflammation in Neurodegeneration, 140 (2010) 918–934. doi:10.1016/j.cell.2010.02.016.Mechanisms.

[64] M. Fuhrmann, T. Bittner, C.K.E. Jung, S. Burgold, R.M. Page, G. Mitteregger, C. Haass, F.M. LaFerla, H. Kretzschmar, J. Herms, Microglial Cx3cr1 knockout prevents neuron loss in a mouse model of Alzheimer´s disease, 13 (2010) 411–413. doi:10.1038/nn.2511.Microglial.

[65] M. Prince, E. Albanese, M. Guerchet, M. Prina, World Alzheimer Report 2014 Dementia and Risk Reduction An AnAlysis of pRotective AnD moDifiAble fActoRs EXECUTIVE SUMMARY dr Maëlenn Guerchet dr Matthew prina, (2014).

[66] C.A. Lane, Alzheimer's disease, Eur. J. Neurol. 1 (2017) 59–70. doi:10.1111/ene.13439.

[67] W. Xu, L. Tan, H.F. Wang, T. Jiang, M.S. Tan, L. Tan, Q.F. Zhao, J.Q. Li, J. Wang, J.T. Yu, Meta-analysis of modifiable risk factors for Alzheimer's disease, J. Neurol. Neurosurg. Psychiatry. 86 (2015) 1299–1306. doi:10.1136/jnnp-2015-310548.

[68] H.W. Querfurth, F.M. Laferla, Alzheimer's Disease, 9 (2010) 329–344.

[69] L.F. Lue, D.G. Walker, J. Rogers, Modeling microglial activation in Alzheimer's disease with human postmortem microglial cultures, Neurobiol. Aging. 22 (2001) 945–956. doi:10.1016/S0197-4580(01)00311-6.

[70] T. Nagae, K. Araki, Y. Shimoda, L. Sue, T. Beach, Y. Konishi, Cytokines and Cytokine Receptors Involved in the Pathogenesis of Alzheimer´s Disease, 2016. doi:10.4172/2155-9899.1000441.

[71] T. Minett, J. Classey, F.E. Matthews, M. Fahrenhold, M. Taga, C. Brayne, P.G. Ince, J.A.R. Nicoll, D. Boche, Microglial immunophenotype in dementia with Alzheimer's pathology, J. Neuroinflammation. 13 (2016) 1–10. doi:10.1186/s12974-016-0601-z.

[72] K.L. Kumawat, D.K. Kaushik, P. Goswami, A. Basu, Acute exposure to lead acetate activates microglia and induces subsequent bystander neuronal death via caspase-3 activation, Neurotoxicology. 41 (2014) 143–153. doi:10.1016/j.neuro.2014.02.002.

[73] K. Wilkinson, J. El Khoury, Microglial scavenger receptors and their roles in the pathogenesis of Alzheimer's disease, Int. J. Alzheimers. Dis. 2012 (2012) 1–10. doi:10.1155/2012/489456.

[74] L. Hirsch, N. Jette, A. Frolkis, T. Steeves, T. Pringsheim, The Incidence of Parkinson's Disease: A Systematic Review and Meta-Analysis, Neuroepidemiology. 46 (2016) 292–300. doi:10.1159/000445751.

[75] C. Lill, Genetics of Parkinson disease, Mol. Cell. Probes. 30 (2016) 386–396. doi:10.1016/B978-0-444-63233-3.00014-2.

[76] H.-M. Gao, J. Hong, Gene–environment interactions: key to unraveling the mystery of Parkinson's disease, 94 (2011) 1–19. doi:10.1038/mp.2011.182.doi.

[77] D.W. Dickson, H. Braak, J.E. Duda, C. Duyckaerts, T. Gasser, G.M. Halliday, J. Hardy, J.B. Leverenz, K. Del Tredici, Z.K. Wszolek, I. Litvan, Neuropathological assessment of Parkinson's disease: refining the diagnostic criteria, Lancet Neurol. 8 (2009) 1150–1157. doi:10.1016/S1474-4422(09)70238-8.

[78] M.W. Dodson, M. Guo, Pink1, Parkin, DJ-1 and mitochondrial dysfunction in Parkinson's disease, Curr. Opin. Neurobiol. 17 (2007) 331–337. doi:10.1016/j.conb.2007.04.010.

[79] S. Saha, M. Guillily, A. Ferree, J. Lanceta, D. Chan, J. Ghosh, C. Hsu, L. Segal, K. Raghavam, K. Matsumoto, N. Hisamoto, T. Kuwahara, T. Iwatsubo, L. Moore, L. Goldstein, M. Cookson, B. Wolozin, LRRK2 modulates vulnerability to mitochondrial dysfunction in C. elegans, J. Neurosci. 29 (2009) 9210–9218. doi:10.1523/JNEUROSCI.2281-09.2009.

[80] V. Bonifati, P. Rizzu, F. Squitieri, E. Krieger, N. Vanacore, J.C. van Swieten, A. Brice, C.M. van Duijn, B. Oostra, G. Meco, P. Heutink, DJ-1 (PARK7), a novel gene for autosomal recessive, early onset parkinsonism, Neurol. Sci. 24 (2003) 159–160. doi:10.1007/s10072-003-0108-0.

[81] J. Kim, J.-W. Byun, I. Choi, B. Kim, H.-K. Jeong, I. Jou, E. Joe, PINK1 Deficiency Enhances Inflammatory Cytokine Release from Acutely Prepared Brain Slices, Exp. Neurobiol. 22 (2013) 38–44. doi:10.5607/en.2013.22.1.38.

[82] B. Kim, M.S. Yang, D. Choi, J.H. Kim, H.S. Kim, W. Seol, S. Choi, I. Jou, E.Y. Kim, E. hye Joe, Impaired inflammatory responses in murine lrrk2-knockdown brain microglia, PLoS One. 7 (2012) 1–12. doi:10.1371/journal.

pone.0034693.

[83] A. Al-Chalabi, O. Hardiman, The epidemiology of ALS: a conspiracy of genes, environment and time., Nat. Rev. Neurol. 9 (2013) 617–628. doi:10.1038/nrneurol.2013.203.

[84] S. Rossi, M. Cozzolino, M.T. Carrì, Old versus new mechanisms in the pathogenesis of ALS, Brain Pathol. 26 (2016) 276–286. doi:10.1111/bpa.12355.

[85] N. D'Ambrosi, P. Finocchi, S. Apolloni, M. Cozzolino, A. Ferri, V. Padovano, G. Pietrini, M.T. Carri, C. Volonte, The Proinflammatory Action of Microglial P2 Receptors Is Enhanced in SOD1 Models for Amyotrophic Lateral Sclerosis, J. Immunol. 183 (2009) 4648–4656. doi:10.4049/jimmunol.0901212.

[86] J.S. Henkel, D.R. Beers, W. Zhao, S.H. Appel, Microglia in ALS: The good, the bad, and the resting, J. Neuroimmune Pharmacol. 4 (2009) 389–398. doi:10.1007/s11481-009-9171-5.

[87] P. Corcia, C. Tauber, J. Vercoullie, N. Arlicot, C. Prunier, J. Praline, G. Nicolas, Y. Venel, C. Hommet, J.L. Baulieu, J.P. Cottier, C. Roussel, M. Kassiou, D. Guilloteau, M.J. Ribeiro, Molecular Imaging of Microglial Activation in Amyotrophic Lateral Sclerosis, PLoS One. 7 (2012) 6–12. doi:10.1371/journal.pone.0052941.

[88] S.S. Chaganti, E.A. McCusker, C.T. Loy, What do we know about late onset Huntington's disease?, J. Huntingtons. Dis. 6 (2017) 95–103. doi:10.3233/JHD-170247.

[89] S.D. Kim, V.S.C. Fung, An update on Huntington's disease: From the gene to the clinic, Curr. Opin. Neurol. 27 (2014) 477–483. doi:10.1097/WCO.0000000000000116.

[90] F.O. Walker, Huntington's disease, Lancet. 369 (2007) 218–228. doi:10.1016/S0140-6736(07)60111-1.

[91] H.M. Yang, S. Yang, S.S. Huang, B.S. Tang, J.F. Guo, Microglial activation in the pathogenesis of Huntington's Disease, Front. Aging Neurosci. 9 (2017) 1–9. doi:10.3389/fnagi.2017.00193.

[92] P. McColgan, S.J. Tabrizi, Huntington's disease: a clinical review, Eur. J. Neurol. 25 (2018) 24–34. doi:10.1111/ene.13413.

[93] S.K. Singhrao, J.W. Neal, B.P. Morgan, P. Gasque, Increased complement biosynthesis by microglia and complement activation on neurons in Huntington's disease, Exp. Neurol. 159 (1999) 362–376. doi:10.1006/exnr.1999.7170.

[94] G. Ellrichmann, C. Reick, C. Saft, R.A. Linker, The Role of the Immune System in Huntington's Disease, Clin. Dev. Immunol. 541259 (2013) 1–11. doi:10.1155/2013/541259.

[95] E. Wild, A. Magnusson, N. Lahiri Swales, U. Krus, M. Orth, S.J. Tabrizi, M. Björkqvist, Abnormal peripheral chemokine profile in Huntington's disease, PLoS Curr. RRN1231 (2011) 1–11. doi:10.1371/currents.RRN1231.

[96] M. Björkqvist, Immunomodulation - a disease-modifying avenue for treatment of Huntington's disease?, J. Neurochem. 137 (2016) 670–672. doi:10.1111/jnc.13539.

[97] T. Zrzavy, S. Hametner, I. Wimmer, O. Butovsky, H.L. Weiner, H. Lassmann, Loss of "homeostatic" microglia and patterns of their activation in active multiple sclerosis, Brain. 140 (2017) 1900–1913. doi:10.1093/brain/awx113.

[98] D. Lall, R.H. Baloh, Microglia and C9orf72 in neuroinflammation and ALS and frontotemporal dementia, J. Clin. Invest. 127 (2017) 3250–3258. doi:10.1172/JCI90607.

[99] P. Cheepsunthorn, L. Radov, S. Menzies, J. Reid, J.R. Connor, Characterization of a novel brain-derived microglial cell line isolated from neonatal rat brain, Glia. 35 (2001) 53–62. doi:10.1002/glia.1070.

[100] E. Blasi, R. Barluzzi, F. Bistoni, Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus, J. Neuroimmunol. 27 (1990) 229–237. doi:10.1016/0165-5728(90)90073-V.

[101] B. Stansley, J. Post, K. Hensley, A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease, J. Neuroinflammation. 9 (2012) 1–8. doi:10.1186/1742-2094-9-115.

[102] F. Gao, D. Chen, Q. Hu, G. Wang, Rotenone Directly Induces BV2 Cell Activation via the p38 MAPK Pathway, PLoS One. 8 (2013) 1–9. doi:10.1371/journal.pone.0072046.

[103] A. Griciuc, A. Serrano, A. Parrado, A. Lesinski, C. Asselin, K. Mullin, B. Hooli, S. Choi, B. Hyman, R. Tanzi, Alzheimer´s disease risk gene CD33 inhibits microglial uptake of amyloid beta, Neuron. 78 (2013) 631–643. doi:10.1016/j.neuron.2013.04.014.

[104] A. Nagai, E. Nakagawa, K. Hatori, H.B. Choi, J.G. McLarnon, M.A. Lee, S.U. Kim, Generation and characterization of immortalized human microglial cell lines: Expression of cytokines and chemokines, Neurobiol. Dis. 8 (2001) 1057–1068. doi:10.1006/nbdi.2001.0437.

[105] I. Neiva, J.O. Malva, J. Valero, Can we talk about microglia without neurons? A discussion of microglial cell autonomous properties in culture, Front. Cell. Neurosci. 8 (2014) 1–5. doi:10.3389/fncel.2014.00202.

[106] L. Xue, H. Yi, Z. Huang, Y.B. Shi, W.X. Li, Global gene expression during the human organogenesis: From tran-scription profiles to function predictions, Int. J. Biol. Sci. 7 (2011) 1068–1076. doi:10.7150/ijbs.7.1068.

[107] T.F. Galatro, I.R. Holtman, A.M. Lerario, I.D. Vainchtein, N. Brouwer, P.R. Sola, M.M. Veras, T.F. Pereira, R.E.P. Leite, T. Möller, P.D. Wes, M.C. Sogayar, J.D. Laman, W. Den Dunnen, C.A. Pasqualucci, S.M. Oba-shinjo, E.W.G.M. Boddeke, S.K.N. Marie, B.J.L. Eggen, Transcriptomic analysis of purified human cortical microglia reveals age-associated changes, Nat. Publ. Gr. (2017). doi:10.1038/nn.4597.

[108] P. Saenger, Turner's Syndrome, N. Engl. J. Med. 335 (1996) 1749–1754. doi:10.1056/NEJM199612053352307.

[109] N. Chester, F. Kuo, C. Kozak, C.D. O'Hara, P. Leder, Stage-specific apoptosis, developmental delay, and embryonic lethality in mice homozygous for a targeted disruption in the murine Bloom's syndrome gene, Genes Dev. 12 (1998) 3382–3393. doi:10.1101/gad.12.21.3382.

[110] D. Giulian, T. Baker, Characterization of ameboid microglia isolated from developing mammalian brain, J. Neurosci. 6 (1986) 2163–2178. doi:10.1523/jneurosci.06-08-02163.1986.

[111] T.F. Galatro, I.R. Holtman, A.M. Lerario, I.D. Vainchtein, N. Brouwer, P.R. Sola, M.M. Veras, T.F. Pereira, R.E.P. Leite, T. Möller, P.D. Wes, M.C. Sogayar, J.D. Laman, W. Den Dunnen, C.A. Pasqualucci, S.M. Oba-Shinjo, E.W.G.M. Boddeke, S.K.N. Marie, B.J.L. Eggen, Transcriptomic analysis of purified human cortical microglia reveals age-associated changes, Nat. Neurosci. (2017). doi:10.1038/nn.4597.

[112] B.A. Durafourt, C.S. Moore, M. Blain, J.P. Antel, Isolating, Culturing, and Polarizing Primary Human Adult and Fetal Microglia, Encycl. Neurol. Sci. 1041 (2014) 199–211. doi:10.1016/B978-0-12-385157-4.00184-6.

[113] J. Melief, M.A.M. Sneeboer, M. Litjens, P.R. Ormel, S.J.M.C. Palmen, I. Huitinga, R.S. Kahn, E.M. Hol, L.D. de Witte, Characterizing primary human microglia: A comparative study with myeloid subsets and culture models, Glia. 64 (2016) 1857–1868. doi:10.1002/glia.23023.

[114] M.R. Mizee, M. van der Poel, I. Huitinga, Purification of cells from fresh human brain tissue: primary human glial cells, 1st ed., Elsevier B.V., 2018. doi:10.1016/B978-0-444-63639-3.00019-0.

[115] L.R. Watkins, M.R. Hutchinson, A concern on comparing 'apples' and 'oranges' when differences between microglia used in human and rodent studies go far, far beyond simply species: comment on Smith and Dragunow, Trends Neurosci. 37 (2014) 189–190. doi:10.1016/j.tins.2014.02.004.

[116] J. Rogers, D. Mastroeni, B. Leonard, J. Joyce, A. Grover, Neuroinflammation in Alzheimer's Disease and Parkinson's Disease: Are Microglia Pathogenic in Either Disorder?, Int. Rev. Neurobiol. 82 (2007) 235–246. doi:10.1016/S0074-7742(07)82012-5.

[117] E. Drummond, T. Wisniewski, Alzheimer's disease: experimental models and reality, Acta Neuropathol. (2017). doi:10.1007/s00401-016-1662-x.

[118] T. Tomiyama, S. Matsuyama, H. Iso, T. Umeda, H. Takuma, K. Ohnishi, K. Ishibashi, R. Teraoka, N. Sakama, T. Yamashita, K. Nishitsuji, K. Ito, H. Shimada, M.P. Lambert, W.L. Klein, H. Mori, A mouse model of amyloid β oligomers: Their contribution to synaptic alteration, abnormal tau phosphorylation, glial activation, and neuronal loss in vivo, J. Neurosci. (2010). doi:10.1523/JNEUROSCI.5825-09.2010.

[119] J. Lewis, E. McGowan, J. Rockwood, H. Melrose, P. Nacharaju, M. Van Slegtenhorst, K. Gwinn-Hardy, M.P. Murphy, M. Baker, X. Yu, K. Duff, J. Hardy, A. Corral, W.L. Lin, S.H. Yen, D.W. Dickson, P. Davies, M. Hutton, Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L)tau protein, Nat. Genet. 25 (2000) 402–405. doi:10.1038/78078.

[120] C.J. Bohlen, F.C. Bennett, A.F. Tucker, H.Y. Collins, S.B. Mulinyawe, B.A. Barres, Diverse Requirements for Microglial Survival, Specification, and Function Revealed by Defined-Medium Cultures, Neuron. (2017). doi:10.1016/j.neuron.2017.04.043.

[121] P.J. Roqué, L.G. Costa, Co-Culture of Neurons and Microglia, Curr. Protoc. Toxicol. (2017). doi:10.1002/cptx.32.

[122] C.L. Croft, W. Noble, Preparation of organotypic brain slice cultures for the study of Alzheimer's disease, F1000Research. (2018). doi:10.12688/f1000research.14500.2.

[123] J.W. Linsley, A. Tripathi, I. Epstein, G. Schmunk, E. Mount, M. Campioni, V. Oza, M. Barch, A. Javaherian, T.J. Nowakowski, S. Samsi, S. Finkbeiner, Automated four-dimensional long term imaging enables single cell tracking within organotypic brain slices to study neurodevelopment and degeneration, Commun. Biol. (2019). doi:10.1038/s42003-019-0411-9.

[124] A. Daria, A. Colombo, G. Llovera, H. Hampel, M. Willem, A. Liesz, C. Haass, S. Tahirovic, Young microglia restore amyloid plaque clearance of aged microglia, EMBO J. (2017). doi:10.15252/embj.201694591.

[125] A.M. Benediktsson, S.J. Schachtele, S.H. Green, M.E. Dailey, Ballistic labeling and dynamic imaging of astrocytes in organotypic hippocampal slice cultures, J. Neurosci. Methods. (2005). doi:10.1016/j.jneumeth.2004.05.013.

[126] M. Czapiga, C.A. Colton, Function of microglia in organotypic slice cultures, J. Neurosci. Res. (1999). doi:10.1002/(SICI)1097-4547(19990615)56:6<644::AID-JNR10>3.0.CO;2-9.

[127] E.E. Spangenberg, K.N. Green, Inflammation in Alzheimer's disease: Lessons learned from microglia-depletion models, Brain. Behav. Immun. (2017). doi:10.1016/j.bbi.2016.07.003.

[128] H. Akiyama, S. Barger, S. Barnum, B. Bradt, J. Bauer, G.M. Cole, N.R. Cooper, P. Eikelenboom, M. Emmerling, B.L. Fiebich, C.E. Finch, S. Frautschy, W.S. Griffin, H. Hampel, M. Hull, G. Landreth, L. Lue, R. Mrak, I.R. Mackenzie, P.L. McGeer, M.K. O'Banion, J. Pachter, G. Pasinetti, C. Plata-Salaman, J. Rogers, R. Rydel, Y. Shen, W. Streit, R. Strohmeyer, I. Tooyoma, F.L. Van Muiswinkel, R. Veerhuis, D. Walker, S. Webster, B. Wegrzyniak, G. Wenk, T. Wyss-Coray, Inflammation and Alzheimer's disease, Neurobiol. Aging. 21 (2000) 383–421. doi:10.1016/s0197-4580(00)00124-x.

[129] L. Stoppini, P.A. Buchs, D. Muller, A simple method for organotypic cultures of nervous tissue, J. Neurosci. Methods. (1991). doi:10.1016/0165-0270(91)90128-M.

[130] B.H. Gähwiler, M. Capogna, D. Debanne, R.A. McKinney, S.M. Thompson, Organotypic slice cultures: A technique has come of age, Trends Neurosci. (1997). doi:10.1016/S0166-2236(97)01122-3.

[131] A. Masuch, R. van der Pijl, L. Füner, Y. Wolf, B. Eggen, E. Boddeke, K. Biber, Microglia replenished OHSC: A culture system to study in vivo like adult microglia, Glia. (2016). doi:10.1002/glia.23002.

[132] J. Han, R.A. Harris, X.-M. Zhang, An updated assessment of microglia depletion: current concepts and future directions, Mol. Brain. 10 (2017) 25. doi:10.1186/s13041-017-0307-x.

[133] S. Jung, J. Aliberti, P. Graemmel, M.J. Sunshine, G.W. Kreutzberg, A. Sher, D.R. Littman, Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion, Mol. Cell. Biol. 20 (2000) 4106–4114. doi:10.1128/mcb.20.11.4106-4114.2000.

[134] A. Kohl, F. Dehghani, H.W. Korf, N.P. Hailer, The bisphosphonate clodronate depletes microglial cells in excitotoxically injured organotypic hippocampal slice cultures, Exp. Neurol. (2003). doi:10.1016/S0014-4886(02)00049-3.

[135] J. Vinet, H.R.J. van Weering, A. Heinrich, R.E. Kälin, A. Wegner, N. Brouwer, F.L. Heppner, N. van Rooijen, H.W.G.M. Boddeke, K. Biber, Neuroprotective function for ramified microglia in hippocampal excitotoxicity, J. Neuroinflammation. (2012). doi:10.1186/1742-2094-9-27.

[136] S. Hellwig, A. Masuch, S. Nestel, N. Katzmarski, M. Meyer-Luehmann, K. Biber, Forebrain microglia from wild-type but not adult 5xFAD mice prevent amyloid-β plaque formation in organotypic hippocampal slice cultures, Sci. Rep. (2015). doi:10.1038/srep14624.

[137] K. Ji, G. Akgul, L.P. Wollmuth, S.E. Tsirka, Microglia Actively Regulate the Number of Functional Synapses, PLoS One. (2013). doi:10.1371/journal.pone.0056293.

[138] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell. 126 (2006) 663–676. doi:10.1016/j.cell.2006.07.024.

[139] F. Soldner, J. Laganière, A.W. Cheng, D. Hockemeyer, Q. Gao, R. Alagappan, V. Khurana, L.I. Golbe, R.H. Myers, S. Lindquist, L. Zhang, D. Guschin, L.K. Fong, B.J. Vu, X. Meng, F.D. Urnov, E.J. Rebar, P.D. Gregory, H.S. Zhang, R. Jaenisch, Generation of isogenic pluripotent stem cells differing exclusively at two early onset parkinson point mutations, Cell. 146 (2011) 318–331. doi:10.1016/j.cell.2011.06.019.

[140] D. Hockemeyer, R. Jaenisch, Induced pluripotent stem cells meet genome editing, Cell Stem Cell. 18 (2016) 573–586. doi:10.1016/j.stem.2016.04.013.

[141] Y. Avior, I. Sagi, N. Benvenisty, Pluripotent stem cells in disease modelling and drug discovery, Nat. Rev. Mol. Cell Biol. 17 (2016) 170–182. doi:10.1038/nrm.2015.27.

[142] J.L. Sterneckert, P. Reinhardt, H.R. Schöler, Investigating human disease using stem cell models, Nat. Rev. Genet. 15 (2014) 625–639. doi:10.1038/nrg3764.

[143] J. Mertens, D. Reid, Y. Kim, F. Gage, Aging in a Dish: iPSC-Derived and Directly Induced Neurons for Studying Brain Aging and Age-Related Neurodegenerative Diseases, Annu Rev Genet. 54 (2018) 271–293. doi:10.1146/annurev-genet-120417-031534.

[144] J. Mertens, A.C.M. Paquola, M. Ku, E. Hatch, L. Böhnke, S. Ladjevardi, S. McGrath, B. Campbell, H. Lee, J.R. Herdy, J.T. Gonçalves, T. Toda, Y. Kim, J. Winkler, J. Yao, M.W. Hetzer, F.H. Gage, Directly Reprogrammed Human Neurons Retain Aging- Associated Transcriptomic Signatures and Reveal Age-Related Nucleocytoplasmic Defects Jerome, Cell Stem Cell. 17 (2015) 705–718. doi:10.1097/CCM.0b013e31823da96d.Hydrogen.

[145] K. Kim, R. Zhao, A. Doi, K. Ng, J. Unternaehrer, P. Cahan, H. Hongguang, Y. Loh, M.J. Aryee, M.W. Lensch, H. Li, J.J. Collins, A.P. Feinberg, G.Q. Daley, Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells, Nat. Rev Drug Discov. 29 (2012) 1117–1119. doi:10.4172/2157-7633.1000305.Improved.

[146] Y. Ohi, H. Qin, C. Hong, L. Blouin, J.M. Polo, T. Guo, Z. Qi, S.L. Downey, P.D. Manos, D.J. Rossi, J. Yu, M. Hebrok, K. Hochedlinger, J.F. Costello, J.S. Song, M. Ramalho-Santos, Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells, Nat. Cell Biol. 13 (2011) 541–549. doi:10.1038/ncb2239.

[147] O. Bar-Nur, H.A. Russ, S. Efrat, N. Benvenisty, Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells, Cell Stem Cell. 9 (2011) 17–23. doi:10.1016/j.stem.2011.06.007.

[148] A. Prasad, J. Manivannan, P.M. Gharibani, D.T. Loong, S.M. Chua, A.H. All, A review of induced pluripotent stem cell, direct conversion by trans-differentiation, direct reprogramming and oligodendrocyte differentiation, Regen. Med. 11 (2016) 181–191. doi:10.2217/rme.16.5.

[149] H.N. Nguyen, B. Byers, B. Cord, A. Shcheglovitov, J. Byrne, P. Gujar, K. Kee, B. Schule, R.E. Dolmetsch, W. Langston, T. Palmer, R.R. Pera, LRRK2 Mutant iPSC-Derived DA Neurons Demonstrate Increased Susceptibility to Oxidative Stress, Cell Stem Cell. 8 (2011) 267–280. doi:10.1371/journal.pone.0178059.

[150] O. Cooper, et al, Pharmacological Rescue of Mitochondrial Deficits in iPSC-Derived Neural Cells from Patients with Familial Parkinson's Disease, Sci. Transl. Med. 4 (2012) 1–13. doi:10.1126/scitranslmed.3003985.

[151] R. Pollex, R. Hegele, Hutchinson-Gilford progeria syndrome, Clin. Genet. 66 (2004) 375–381. doi:10.1016/B978-0-444-62702-5.00018-4.

[152] J.D. Miller, Y.M. Ganat, S. Kishinevsky, R.L. Bowman, B. Liu, E.Y. Tu, P.K. Mandal, E. Vera, J.W. Shim, S. Kriks, T. Taldone, N. Fusaki, M.J. Tomishima, D. Krainc, T.A. Milner, D.J. Rossi, L. Studer, Human iPSC-based modeling of late-onset disease via progerin-induced aging, Cell Stem Cell. 13 (2013) 691–705. doi:10.1016/j.stem.2013.11.006.

[153] C.A. Ross, S.S. Akimov, Human-induced pluripotent stem cells: Potential for neurodegenerative diseases, Hum. Mol. Genet. 23 (2014) 17–26. doi:10.1093/hmg/ddu204.

[154] J. Muffat, Y. Li, B. Yuan, M. Mitalipova, A. Omer, S. Corcoran, G. Bakiasi, L.-H. Tsai, P. Aubourg, R.M. Ransohoff, others, Efficient derivation of microglia-like cells from human pluripotent stem cells, Nat. Med. 22 (2016) 1358.

[155] E.M. Abud, R.N. Ramirez, E.S. Martinez, M.J. Carson, W.W. Poon, M. Blurton-jones, E.M. Abud, R.N. Ramirez, E.S. Martinez, L.M. Healy, C.H.H. Nguyen, S.A. Newman, iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases, Neuron. 94 (2017) 278-293.e9. doi:10.1016/j.neuron.2017.03.042.

[156] P. Douvaras, B. Sun, M. Wang, I. Kruglikov, G. Lallos, M. Zimmer, C. Terrenoire, B. Zhang, S. Gandy, E. Schadt, D.O. Freytes, S. Noggle, V. Fossati, Directed Differentiation of Human Pluripotent Stem Cells to Microglia, Stem Cell Reports. (2017). doi:10.1016/j.stemcr.2017.04.023.

[157] W. Haenseler, S.N. Sansom, J. Buchrieser, S.E. Newey, C.S. Moore, F.J. Nicholls, S. Chintawar, C. Schnell, J.P. Antel, N.D. Allen, M.Z. Cader, R. Wade-martins, W.S. James, S.A. Cowley, A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response, Stem Cell Reports. 8 (2017) 1727–1742. doi:10.1016/j.stemcr.2017.05.017.

[158] H. Pandya, M.J. Shen, D.M. Ichikawa, A.B. Sedlock, Y. Choi, K.R. Johnson, G. Kim, M.A. Brown, A.G. Elkahloun, D. Maric, others, Differentiation of human and murine induced pluripotent stem cells to microglia-like cells, Nat. Neurosci. 20 (2017) 753.

[159] P.W. Brownjohn, J. Smith, R. Solanki, E. Lohmann, H. Houlden, J. Hardy, S. Dietmann, F.J. Livesey, Functional Studies of Missense TREM2 Mutations in Human Stem Cell-Derived Microglia, Stem Cell Reports. 10 (2018) 1294–1307. doi:10.1016/j.stemcr.2018.03.003.

[160] P. Garcia-Reitboeck, A. Phillips, T.M. Piers, C. Villegas-Llerena, M. Butler, A. Mallach, C. Rodrigues, C.E. Arber, A. Heslegrave, H. Zetterberg, H. Neumann, S. Neame, H. Houlden, J. Hardy, J.M. Pocock, Human Induced Pluripotent Stem Cell-Derived Microglia-Like Cells Harboring TREM2 Missense Mutations Show Specific Deficits in Phagocytosis, Cell Rep. 24 (2018) 2300–2311. doi:10.1016/j.celrep.2018.07.094.

[161] A. McQuade, M. Coburn, C.H. Tu, J. Hasselmann, H. Davtyan, M. Blurton-Jones, Development and validation of a simplified method to generate human microglia from pluripotent stem cells, Mol. Neurodegener. 13 (2018) 1–13. doi:10.1186/s13024-018-0297-x.

[162] D.A. Lim, A.D. Tramontin, J.M. Trevejo, D.G. Herrera, J.M. García-Verdugo, A. Alvarez-Buylla, Noggin antagonizes BMP signaling to create a niche for adult neurogenesis, Neuron. 28 (2000) 713–726. doi:10.1016/S0896-6273(00)00148-3.

[163] E. Porlan, J.M. Morante-Redolat, M.Á. Marqués-Torrejón, C. Andreu-Agulló, C. Carneiro, E. Gómez-Ibarlucea, A. Soto, A. Vidal, S.R. Ferrón, I. Fariñas, Transcriptional repression of Bmp2 by p21 Waf1/Cip1 links quiescence to neural stem cell maintenance, Nat. Neurosci. 16 (2013) 1567–1575. doi:10.1038/nn.3545.

[164] A.M. Smith, H.M. Gibbons, R.L. Oldfield, P.M. Bergin, E.W. Mee, R.L.M. Faull, M. Dragunow, The tran-

scription factor PU.1 is critical for viability and function of human brain microglia, Glia. 61 (2013) 929–942. doi:10.1002/glia.22486.

[165] D. Ito, Microglia-specific localisation of a novel calcium binding protein, Iba1, Mol. Brain Res. 57 (1998) 1–9.

[166] C.S. Moore, A.R. Ase, A. Kinsara, V.T.S. Rao, M.M. Robinson, S.Y. Leong, O. Butovsky, S.K. Ludwin, P. Seguela, A. Bar-Or, J.P. Antel, P2Y12 expression and function in alternatively activated human microglia, Neurol. Neuroimmunol. NeuroInflammation. 2 (2015) 1–10. doi:10.1212/NXI.0000000000000080.

[167] S.E. Hickman, N.D. Kingery, T. Oshumi, M. Borowsky, L. Wang, T.K. Means, J.E. Khoury, The Microglial Sensome Revealed by Direct RNA Sequencing, Nat. Neurosci. 16 (2013) 1896–1905. doi:10.1038/nn.3554.The.

[168] Y. Zhang, K. Chen, S.A. Sloan, M.L. Bennett, A.R. Scholze, S. O'Keeffe, H.P. Phatnani, P. Guarnieri, C. Caneda, N. Ruderisch, S. Deng, S.A. Liddelow, C. Zhang, R. Daneman, T. Maniatis, B.A. Barres, J.Q. Wu, An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex, J. Neurosci. 34 (2014) 11929–11947. doi:10.1523/JNEUROSCI.1860-14.2014.

[169] C. Villegas-Llerena, A. Phillips, P.G. Reitboeck, J. Hardy, J.M. Pocock, Microglial genes regulating neuroinflammation in the progression of Alzheimer's disease, Curr. Opin. Neurobiol. 36 (2016) 74–81. doi:10.1016/j.conb.2015.10.004.

[170] H. Asai, S. Ikezu, S. Tsunoda, M. Medalla, J. Luebke, B. Wolozin, O. Butovsky, T. Ikezu, E. Therapeutics, Depletion of microglia and inhibition of exosome synthesis halt tau progagation, 18 (2015) 1584–1593. doi:10.1038/nn.4132.Depletion.

[171] M.L. Bennett, F.C. Bennett, S.A. Liddelow, B. Ajami, J.L. Zamanian, N.B. Fernhoff, S.B. Mulinyawe, C.J. Bohlen, A. Adil, A. Tucker, I.L. Weissman, E.F. Chang, G. Li, G.A. Grant, M.G. Hayden Gephart, B.A. Barres, New tools for studying microglia in the mouse and human CNS., Proc. Natl. Acad. Sci. U. S. A. 113 (2016) E1738–E1746. doi:10.1073/pnas.1525528113.

[172] R.M. Ransohoff, V.H. Perry, Microglial Physiology: Unique Stimuli, Specialized Responses, Annu. Rev. Immunol. 27 (2009) 119–145. doi:10.1146/annurev.immunol.021908.132528.

[173] S.E. Haynes, G. Hollopeter, G. Yang, D. Kurpius, M.E. Dailey, W.B. Gan, D. Julius, The P2Y12 receptor regulates microglial activation by extracellular nucleotides, Nat. Neurosci. 9 (2006) 1512–1519. doi:10.1038/nn1805.

[174] B. van Wilgenburg, C. Browne, J. Vowles, S.A. Cowley, Efficient, Long Term Production of Monocyte-Derived Macrophages from Human Pluripotent Stem Cells under Partly-Defined and Fully-Defined Conditions, PLoS One. 8 (2013) 1–18. doi:10.1371/journal.pone.0071098.

[175] S.M. Hancock, D.I. Finkelstein, P.A. Adlard, Glia and zinc in ageing and alzheimer's disease: A mechanism for cognitive decline?, Front. Aging Neurosci. 6 (2014) 1–6. doi:10.3389/fnagi.2014.00137.

[176] H. Konttinen, M. e C. Cabral-da-Silva, S. Ohtonen, S. Wojciechowski, A. Shakirzyanova, S. Caligola, R. Giugno, Y. Ishchenko, D. Hernández, M.F. Fazaludeen, S. Eamen, M.G. Budia, I. Fagerlund, F. Scoyni, P. Korhonen, N. Huber, A. Haapasalo, A.W. Hewitt, J. Vickers, G.C. Smith, M. Oksanen, C. Graff, K.M. Kanninen, S. Lehtonen, N. Propson, M.P. Schwartz, A. Pébay, J. Koistinaho, L. Ooi, T. Malm, PSEN1ΔE9, APPswe, and APOE4 Confer Disparate Phenotypes in Human iPSC-Derived Microglia, Stem Cell Reports. 13 (2019) 669–683. doi:https://doi.org/10.1016/j.stemcr.2019.08.004.

[177] K. Saijo, C.K. Glass, Microglial cell origin and phenotypes in health and disease, Nat. Rev. Immunol. 11 (2011) 775–787. doi:10.1038/nri3086.

[178] O. Butovsky, M.P. Jedrychowski, C.S. Moore, R. Cialic, A.J. Lanser, G. Gabriely, T. Koeglsperger, B. Dake, P.M. Wu, C.E. Doykan, Z. Fanek, L. Liu, Z. Chen, J.D. Rothstein, R.M. Ransohoff, S.P. Gygi, J.P. Antel, H.L. Weiner, Identification of a unique TGF-β-dependent molecular and functional signature in microglia, Nat. Neurosci. 17 (2014) 131–143. doi:10.1038/nn.3599.

[179] F. Antonica, D.F. Kasprzyk, R. Opitz, M. Iacovino, X.-H. Liao, M. Dumitresco, S. Refetoff, K. Peremans, M. Manto, M. Kyba, S. Costagliola, Generation of functional thyroid from embryonic stem cells, Thyroid Dis. Child. Recent Adv. from Basic Sci. to Clin. Pract. 491 (2012) 66–71. doi:10.1007/978-3-319-19213-0_19.

[180] H. Suga, T. Kadoshima, M. Minaguchi, M. Ohgushi, M. Soen, T. Nakano, N. Takata, T. Wataya, K. Muguruma, H. Miyoshi, S. Yonemura, Y. Oiso, Y. Sasai, Self-formation of functional adenohypophysis in three-dimensional culture, Nature. 480 (2011) 57–62. doi:10.1038/nature10637.

[181] A. Fatehullah, S.H. Tan, N. Barker, Organoids as an in vitro model of human development and disease, Nat. Cell Biol. 18 (2016) 246–254. doi:10.1038/ncb3312.

[182] M.A. Lancaster, M. Renner, C.-A. Martin, D. Wenzel, L.S. Bicknell, M.E. Hurles, T. Homfray, J.M. Penninger, A.P. Jackson, J.A. Knoblich, Cerebral organoids model human brain development and microcephaly., Nature. 501 (2013) 373–379. doi:10.1038/nature12517.

[183] X. Qian, H.N. Nguyen, M.M. Song, C. Hadiono, S.C. Ogden, C. Hammack, B. Yao, G.R. Hamersky, F. Jacob, C. Zhong, K.J. Yoon, W. Jeang, L. Lin, Y. Li, J. Thakor, D.A. Berg, C. Zhang, E. Kang, M. Chickering, D. Nauen, C.Y. Ho, Z. Wen, K.M. Christian, P.Y. Shi, B.J. Maher, H. Wu, P. Jin, H. Tang, H. Song, G.L. Ming, Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure, Cell. 165 (2016) 1238–1254. doi:10.1016/j.cell.2016.04.032.

[184] D. Dutta, I. Heo, H. Clevers, Disease Modeling in Stem Cell-Derived 3D Organoid Systems, Trends Mol. Med. 23 (2017) 393–410. doi:10.1016/j.molmed.2017.02.007.

[185] E. Polazzi, A. Contestabile, Reciprocal interactions between microglia and neurons: From survival to neuropathology, Rev. Neurosci. 13 (2002) 221–242. doi:10.1515/REVNEURO.2002.13.3.221.

[186] D. Gosselin, D. Skola, N.G. Coufal, I.R. Holtman, J.C.M. Schlachetzki, E. Sajti, B.N. Jaeger, C. O'Connor, C. Fitzpatrick, M.P. Pasillas, M. Pena, A. Adair, D.D. Gonda, M.L. Levy, R.M. Ransohoff, F.H. Gage, C.K. Glass, An environment-dependent transcriptional network specifies human microglia identity, Science (80-.). 356 (2017) 1248–1259. doi:10.1126/science.aal3222.

[187] A.C.C. da Fonseca, D. Matias, C. Garcia, R. Amaral, L.H. Geraldo, C. Freitas, F.R.S. Lima, The impact of microglial activation on blood-brain barrier in brain diseases, Front. Cell. Neurosci. 8 (2014) 1–13. doi:10.3389/fncel.2014.00362.

[188] M.A. Erickson, W.A. Banks, Blood-brain barrier dysfunction as a cause and consequence of Alzheimer's disease, J. Cereb. Blood Flow Metab. 33 (2013) 1500–1513. doi:10.1038/jcbfm.2013.135.

[189] E. Lyros, C. Bakogiannis, Y. Liu, K. Fassbender, Molecular Links Between Endothelial Dysfunction and Neurodegeneration in Alzheimer's Disease, Curr. Alzheimer Res. 11 (2014) 18–26. doi:10.2174/1567205010666131119235254.

[190] D. Erny, A.L.H. De Angelis, D. Jaitin, P. Wieghofer, O. Staszewski, E. David, H. Keren-Shaul, T. Mahlakoiv, K. Jakobshagen, T. Buch, V. Schwierzeck, O. Utermöhlen, E. Chun, W.S. Garrett, K.D. Mccoy, A. Diefenbach, P. Staeheli, B. Stecher, I. Amit, M. Prinz, Host microbiota constantly control maturation and function of microglia in the CNS, Nat. Neurosci. 18 (2015) 965–977. doi:10.1038/nn.4030.

[191] Y.T. Lin, J. Seo, F. Gao, H.M. Feldman, H.L. Wen, J. Penney, H.P. Cam, E. Gjoneska, W.K. Raja, J. Cheng, R. Rueda, O. Kritskiy, F. Abdurrob, Z. Peng, B. Milo, C.J. Yu, S. Elmsaouri, D. Dey, T. Ko, B.A. Yankner, L.H. Tsai, APOE4 causes widespread molecular and cellular alterations associated with Alzheimer's Disease phenotypes in human iPSC-derived brain cell types, Neuron. 98 (2018) 1141–1154. doi:10.1016/j.neuron.2018.05.008.

[192] P.R. Ormel, R. Vieira de Sá, E.J. van Bodegraven, H. Karst, O. Harschnitz, M.A.M. Sneeboer, L.E. Johansen, R.E. van Dijk, N. Scheefhals, A. Berdenis van Berlekom, E. Ribes Martínez, S. Kling, H.D. MacGillavry, L.H. van den Berg, R.S. Kahn, E.M. Hol, L.D. de Witte, R.J. Pasterkamp, Microglia innately develop within cerebral organoids, Nat. Commun. 9 (2018) 1–14. doi:10.1038/s41467-018-06684-2.

[193] L. Song, X. Yuan, Z. Jones, C. Vied, Y. Miao, M. Marzano, T. Hua, Q.-X.A. Sang, J. Guan, T. Ma, Y. Zhou, Y. Li, Functionalization of Brain Region-specific Spheroids with Isogenic Microglia-like Cells, Sci. Rep. 9 (2019) 11055. doi:10.1038/s41598-019-47444-6.

[194] J. Park, I. Wetzel, I. Marriott, D. Dréau, C. D'Avanzo, D.Y. Kim, R.E. Tanzi, H. Cho, A 3D human triculture system modeling neurodegeneration and neuroinflammation in Alzheimer's disease, Nat. Neurosci. 21 (2018) 941–951. doi:10.1038/s41593-018-0175-4.

CHAPTER 8

Generation and functionality of human microglia-differentiated induced pluripotent stem cells

Angelica Sabogal[1], Arun Thiruvalluvan[2], F. Foijer[3], Bart Eggen[2], Erik Boddeke[2], Amalia Dolga

[1]Dept of Molecular Pharmacology, Faculty of Science and Engineering, Groningen Research Institute of Pharmacy, Behavioral and Cognitive Neurosciences (BCN), University of Groningen, Groningen, The Netherlands

[2]Biomedical Sciences of Cells & Systems, Molecular Neurobiology, Faculty of Medical Sciences, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

[3]European Research Institute for the Biology of Ageing (ERIBA), University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Manuscript in preparation

Abstract

Microglia are the resident macrophages of the brain and are considered the first line of defense against injury in the central nervous system. Microglia play important roles in synaptic plasticity, neurogenesis, brain homeostasis, and neuro-immune activity. They are essentially involved in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease, underlining the need to increase our understanding of their function in disease[1]. Cerebral organoids are human pluripotent stem cell (hPSC)-derived three-dimensional *in vitro* culture systems that can possibly be used as a neural environment to study microglia. To validate this option, the functional and transcriptional similarities between iPSC-derived microglia and microglia co-cultured inside an organoid have to be studied. In our study, the first step was to differentiate microglia from a human iPSC line, according to Douvaras et al., 2017[2] and generate organoids based on Lancaster et al., 2013[3]. We obtained 85% of pure microglial progenitors, positive for CD14 and CX3CR1. We further demonstrated the maturity of these microglia by immunofluorescence staining for CD11b, TMEM119, and Iba-1. To test the functionality of iPSC-derived microglia, we stimulated with LPS and alpha-synuclein and measured real-time cell impedance by xCELLigence and phagocytosis activity using the IncuCyte system. We introduced differentiated microglia to human organoids three-months old. Based on the current data, we conclude that human microglia-differentiated from induced pluripotent stem cells can be used to better understand the microglia function in physiological and pathological conditions.

Keywords: Microglia, brain organoids, Induced pluripotent stem cells

Generation and functionality of human microglia-differentiated induced pluripotent stem cells

Introduction

Microglia are the tissue-resident macrophages of the central nervous system (CNS), with the ability to scavenge dying cells, phagocyte cellular debris, pathogens, and various molecules that are perceived as threatens to the CNS. Microglia is performing these processes using pattern recognition receptors, that are essential for phagocytosis and endocytosis [1,2]. Microglia is part of a group of mononuclear phagocytes that also include peripheral tissue and CNS-associated macrophages, dendritic cells and monocyte cells. During development, microglia have essential roles as effectors and regulators of synaptic plasticity, synaptic pruning neurogenesis, and brain homeostasis, while during aging their neuro-immune activity becomes predominant [3,4]. In the adult mammalian CNS, mature microglia exhibit a small cell soma, with little perinuclear cytoplasm, and highly branched with fine ramified processes covered in small fine protrusions [5]. Microglial activation undergo drastic morphological and functional alterations being classically characterized by two major changes: i) first, the cell shape convert from a highly ramified, motile morphology to a larger, amoeboid form, and ii) second, once in the amoeboid form microglia become active phagocytes [2,6].

Microglial cells are of mesodermal/mesenchymal origin and derive from progenitors that migrated into the CNS from the periphery [1,7]. These invading cells migrate from the extraembryonic yolk sac towards the developing CNS, entering in several migration steps and spots (e.g., the choroid plexus) [8,9]. Notably, upon closure of the blood-brain barrier and cessation of monocyte exchange between the CNS and periphery, and also during adulthood, the number of microglia remains relatively steady based on intrinsic apoptosis and self-renewal [10,11]

The discovery of the four transcription factors Oct4, Sox2, Klf4, and cMyc (known as Yamanaka factors) enables reprogramming of somatic cells (e.g. fibroblasts) into pluripotent stem cells (iPSC). Reprograming somatic cells into iPSC provide an unlimited source of cells while disregarding ethical and practical restrictions [12]. For example, in the field of cell biology, the integration of iPSC-derived cells into 3D brain organoids brings the opportunity to study brain cell interactions and how these interactions affect the pathology of neurodegenerative diseases. In recent years, several protocols became available that allowed for the differentiation of human iPSCs into iPSC-derived microglia (iPSC-MG) [13–18]. A more detailed assessment of iPSC-MGs demonstrated a high congruency with human fetal and adult microglia. First of all, microglia derived from iPSC (iPSC-MG) were found to show similar gene expression when compared to fetal or adult microglia with regard to *P2RY12, GPR34, C1Q, CABLES1, BHLHE41, TREM2, ITAM PROS1, APOE, SLCO2B1, SLC7A8, PPARD*, and *CRYBB1* genes [13]. Moreover, both LPS-induced release of cytokines/chemokines such as TNFα, CCL2, CCL4, and CXCL10 and Ca^{2+} transients in response to ADP highly resembled that of primary microglia responses [13,19]. Functional assessment of iPSC-MG activity

demonstrated their capability of migrating towards the Aβ aggregates and their ability to phagocytose Aβ [13]. Our central aim of this research was to generate iPSC-MG and evaluate their function in a 2D and 3D context (brain organoid).

Materials and Methods

Pluripotent stem cell lines and culture conditions

EH1 and H9 are NIH approved human Pluripotent stem cell lines (PSC) and embryonic stem cell (ESC) lines, respectively. All iPSC lines were derived from skin biopsies of identified donors upon specific institutional review board approvals and informed consent. Stem cell lines were obtained from the European Research Institute for the Biology of Ageing (ERIBA). Pluripotent stem cell lines (PSC) were cultured and expanded onto Matrigel-coated 6well plates in mTeSR1 medium (StemCell Technologies, #05896). Lines were passed after 3-4 days using enzymatic detachment with ReLeSR™ (StemCell Technologies #05872) for 5 min and re-plated in mTeSR1 medium with 10 µM Rock Inhibitor (StemCell Technologies #72302) for 24 hours.

Differentiation of hiPSCs towards to microglia

We followed the protocol developed by Douvaras and colleagues. PSCs were plated onto Matrigel (BD Biosciences) in a 15x10^3 cells/cm^2 density and grown in mTeSR1 medium containing 10µM Rock Inhibitor for 24 hours. When individual colonies reach 80% confluency (2-4 days after plating), differentiation was induced by mTeSR Custom medium (StemCell Technologies), containing 80ng/ml BMP4. mTeSR Custom medium is mTeSR1 medium without Lithium Chloride, GABA, Pipecolic Acid, bFGF, and TGFβ1 (Stem Cell Technologies). The medium was changed daily for 4 days when cells were incubated with StemPro-34 SFM-medium (containing 2mM GutaMAX-I, Life Technologies) supplemented with 25ng/ml bFGF, 100ng/ml SCF and 80ng/ml VEGF. Two days later, the medium was switched to StemPro-34 containing 50ng/ml SCF, 50ng/ml IL-3, 5ng/ml TPO, 50ng/ml M-CSF, and 50ng/ml Flt3 ligand. On day 10, the supernatant fraction of the cultures were pelleted, resuspended in fresh medium (same as before) and returned to their dishes. On day 14, floating cells were pelleted, resuspended in StemPro-34 containing 50ng/ml M-CSF, 50ng/ml Flt3 ligand, and 25ng/ml GM-CSF and replated back to their dishes. The procedure was repeated every four days. From day 24 – 52, a small number of floating cells was processed for flow cytometry analysis to determine the efficiency of microglial progenitor formation regarded as CD14/CX3CR1 double-positive microglial progenitors. After the isolation of CD14+ and CX3CR1+ progenitors, cells were plated onto tissue culture-treated dishes or Thermanox plastic coverslips (all from Thermo Scientific) in a 40- 50x10 ^{3} cells/cm ^{2} in SF–Microglial Medium (RPMI-1640 from Life Technologies supplemented with 2mM GlutaMAX-I, 10ng/ml GM-CSF and 100ng/ml IL-34). Medium was replenished every 3 to 4 days for at least 2 weeks.

Generation and functionality of human microglia-differentiated induced pluripotent stem cells

xCELLigence Impedance-Based System

xCELLigence system Real-Time Cell Analyzer (Roche Diagnostics, Penzberg, Germany) is an instrument with a microelectrodes network in the bottom of the wells culture dish that allows tracking cellular impedance and, in this way, follow cell status including cell number and shape/size. Cell resistance or impedance depicted was indicated as cell index (CI) values and normalization were performed using the RTCA Software 1.2 (ACEA Biosciences) [20]. In our study, cell impedance was used to monitor the real time kinetics of microglial morphology alterations. iPSC-MG were seeded at a density of 15,000 cells/ well in 96-well E-plate (ACEA Biosciences). Previous to plating, background impedance was determined and subtracted as a blank value. Twenty-four hours after plating, the medium for iPSC-MG cells was challenged with 4 conditions: normal medium, 200 ng/mL alpha synuclein (α-Syn) (Cat N. S-1013-1, rPeptide) and 100 or 250 ng/ml LPS (Cat N. L2880, Sigma).

Fluorescence-activated cell sorting (FACS)

Microglial progenitors were incubated with CX3CR1- and/or CD14-conjugated primary antibodies (see antibody table) or their respective isotype controls for 40 min on ice. Cells were then washed in FACS buffer (PBS, 0.5% BSA, 2mM EDTA, 20mM Glucose), pelleted at 300g for 6 min and resuspended in FACS buffer containing DAPI for dead cell exclusion. CD14+ or CD14+/CX3CR1+ cells were isolated via FACS on SH800S cell sorter using the 100µm ceramic nozzle, and 20 psi. Quantification of data was acquired using FlowJo Analysis 0.7 software.

Immunocytochemistry

Mature differentiated iPSC-MG were fixated with 4% paraformaldehyde (PFA) for 25 min. PFA-fixed cells were washed with PBS-A (PBS containing 0.5% Bovine Serum Albumin/BSA) and incubated for 10 min with PBS-T (0.1% Triton X-100), incubated for 2 hours in blocking serum (PBS, 5% BSA and 3% goat serum) and primary antibodies (see table 1) were applied overnight at 4°C. The next day, cells were washed 5x in PBS-A for 5 min, incubated with secondary antibodies for 2 hours at room temperature (RT), washed 3X for 10 min in PBS-A. Coverslips were mounted onto glass slides using Fluoroshield™ with DAPI (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #F6057-20ML). Secondary antibodies were used at 1:200 dilution.

Cortical organoids co-cultured with microglia were fixed in 4% (w/vol) paraformaldehyde for 30 minutes at room temperature and cryoprotected with 30% sucrose (w/vol) in PBS overnight. Organoids were embedded in OCT and eight µm sections were generated. Sections were blocked in 2% normal donkey serum in PBS with 1%BSA prior to immunofluorescent staining. Primary antibodies are described in table 1, and secondary antibodies used were Alexa Fluor-conjugated (Thermo Fisher Scientific). Images were acquired using a Leica SP8 confocal inverted microscope and Leica DM 4000B fluorescence microscope. Fluorescent colors were digitally applied using the Olympus software DP Manager or ImageJ.

Table 1. List of Antibodies used for flow cytometry and immunofluorescent analyses

Name	Host	Brand	Cat No.	Use
IBA1	Rabbit	Wako	019-19741	Immunofluorescence
TMEM119	Rabbit	Sigma	HPA051870	Immunofluorescence
CX3CR1-PE	Mouse	R&D Systems	FAB5204P	FACs
CD14-APC	Mouse	BioRad	MCA596APCT	FACs
B-III Tubulin	Mouse	Santa Cruz	SC-80005	Immunofluorescence
NeuN	Mouse	Millipore	MAB377	Immunofluorescence
KI-67	Rabbit	Abcam	Ab15580	Immunofluorescence
Nestin	Mouse	R&D Systems	MAB1259	Immunofluorescence
GFAP	Rabbit	Dako	Z0334	Immunofluorescence
Vimentin	Goat	Santa Cruz	SC-7557	Immunofluorescence
MAP-2	Mouse	Sigma	M44403	Immunofluorescence
OCT 4	Mouse	Santa Cruz	SC-5279	Immunofluorescence

Phagocytosis assay

Day 10 matured differentiated iPSC-MG were plated into 96-well flat clear bottom black walled polystyrene tissue-culture treated microplates (Essen Bioscience, Cat No.4379, Michigan, US) and allowed to adhere for two hours. 1ug/0.1 ml pHrodo[â] pathogen bioparticles (p35361, Thermo Fischer) were added at indicated concentrations, and the plates were transferred into the IncuCyte ZOOM[â] platform which was housed inside a cell incubator at 37 °C/5% CO_2, until the end of the assay. One image per well were taken every two hours for 24 hours using a 10x objective lens and then analyzed using the IncuCyte TM Basic Software. Red channel acquisition time was 800 ms. An area filter of min 100 max 100 (um^2) was applied. The fluorescence signal was quantified from four technical replicates/ condition applying a mask.

Organoids generation

For cerebral organoid differentiation, we used the protocol from Lancaster et al., 2013. "Briefly, pluripotent stem cells were dissociated from mouse embryonic fibroblasts by dispase treatment followed by trypsinization to generate single cells. In total, 4,500 cells were plated in each well of an ultra-low binding 96-well plate (Corning) in human ES media with low concentration basic fibroblast growth factor (4 ng ml^{-1}) and 50 µM Rho-associated protein kinase (ROCK) inhibitor (Tocris). Embryoid bodies were fed every other day for 6 days then transferred to low-adhesion 24-well plates (Corning) in neural induction media containing Dulbecco's modified eagle medium (DMEM)/F12, 1:100 N2 supplement (Invitrogen), GlutaMAX (Invitrogen), minimum essential media non-essential amino acids (MEM-NEAA) and 1µg ml^{-1} heparin (Sigma). The neuroepithelial tissues were fed every other day for 5 days. On day11, tissues were transferred to droplets of Matrigel (BD Biosciences) by pipetting into cold Matrigel on a sheet of Parafilm with small 3 mm dimples. These droplets were allowed to gel at 37°C, removed from the Parafilm and grown in differentiation media containing a 1:1 mixture of DMEM/F12 and Neurobasal containing

1:200 N2 supplement (Invitrogen), 1:100 B27 supplement without vitamin A (Invitrogen), 3.5 µl l^{-1} 2mercaptoethanol, 1:4,000 insulin (Sigma), 1:100 GlutaMAX (Invitrogen), 1:200 MEM-NEAA. After four days of stationary growth, the droplets were transferred to a spinning bioreactor containing differentiation media as above, except B27 supplement with vitamin A (Invitrogen) was used" [21].

For microglia interaction with organoid studies, on day 98, cortical organoids were transferred individually to single cups of ultra-low attachment. Microglia were passaged with Accutase and resuspended in cortical organoid maturation media and half medium from microglia (RPMI 1640 supplemented with 2mM GlutaMAX-I, 10ng/ml GM-CSF, and 100ng/ml IL-34). 250,000 microglia were added to each organoid. Maturation media was changed every three days after the addition of microglia.

Results

Differentiation of Human iPSCs into microglia-like cells

The well-characterized human iPSC line EH1 and ESC line H9 were obtained from ERIBA Center. We differentiated EH1 and H9 cells into microglial progenitors according to a previously described protocol by Douvaras et al. 2017 (Figure 1.a) [14]. At first, we investigated the morphology of ESC and iPSC, and also the genes expressed in relation to the pluripotency status (Fig Suppl.1). The differentiation was initiated when iPSC or ESC cells reached 80% of confluency (Figure 1.b). iPSC were converted into primitive hemangioblasts induced by bone morphogenetic protein 4 (BMP4) in the medium. As a result of additional factors (e.g. bFGF, SCF, VEGF, IL-3) the primitive hemangioblasts began to develop into microglia progenitors at the day 16-25 (Figure 1.c). At this stage of differentiation, cells from supernatant expressed CD45, while CX3CR1 was upregulated between days 20 and 25, and we observed more floating cells in the medium (Figure 1.d). Interleukin-34 (IL-34) and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation of plated microglial progenitors resulted in mature microglia (Figure 1.e).

We further evaluated the production of microglia progenitors from H9 and EH1 cells. EH1 cells led a higher production of microglial progenitors: 87% compared with 23% from H9 cells (Figure 2.a,d). Microglia phenotype was verified by immunostaining of TMEM 119 for mature microglia and Iba-1 a well-recognized marker for microglia (Figure 2. b-c; e-f). Based on these results, we decided to continue with EH1 cells for the next experiments.

Functional Validation of iPSC differentiated microglia

The generation of iPSC-MG was validated using both morphological and functional assays. Microglia are the brain immune phagocytes with capability to mediate clearance of apoptotic or necrotic cells and removal of unfolded proteins [22]. Besides, microglia

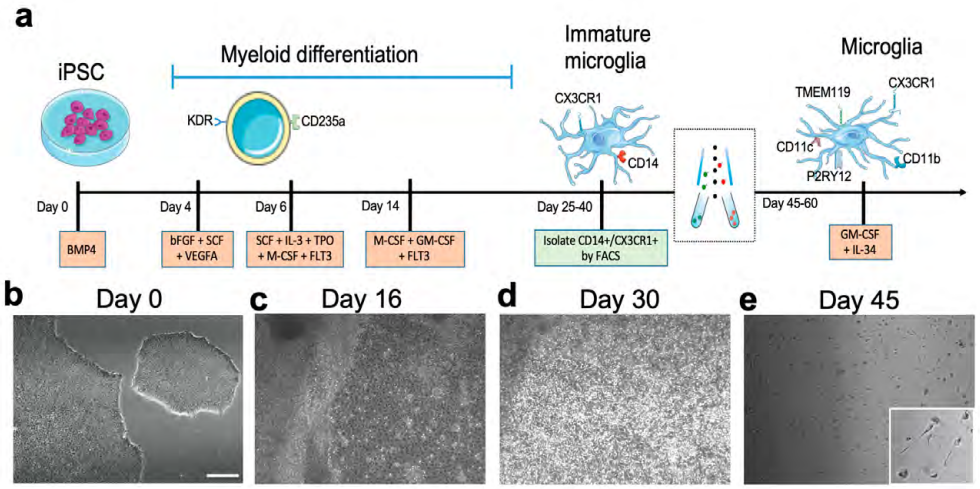


Figure 1. iPSC Differentiation to human microglia. PSC differentiated to microglia through myeloid progenitors. **(a)** Diagram depicting the major steps of the microglial differentiation protocol. **(b-e)** Representative pictures of the main steps in the differentiation protocol. Magnification 4x. Bar graph:100 µm.

contribute to remodeling of neuronal connectivity by engulfment of synapses, axonal and myelin debris and combat central infections by direct phagocytosis of bacteria and viruses [23,24]. Here, we provided evidence of phagocytic activity of iPSC-MG, as a functional readout. We used pHrodo *E. coli* BioParticles conjugates as they are non-fluorescent at neutral pH and they started to show the increase of fluorescent signal (green or red) when the pH is reduced. Acidification of the phagosome content during the phagocytosis process resulted in visualization of red fluorescent particles in active mature microglia (Figure 3. a). Phagocytosis was highly increased by the addition of LPS to the medium (Figure 3.b) as shown by the quantification of mean cell fluorescence intensities of pHrodo *E. coli* BioParticles in iPSC-MG (Figure 3.c). Interestingly, the microglia progenitors are also able to phagocyte pHrodo E. coli BioParticles, while the cell motility seemed reduced compared to mature iPSC-MG (data not shown).

Next, we determined whether microglial cells are able to shift their status from a resting to an activated state, resembling the human microglia and primary microglial ability to become activated. To this end, we challenged iPSC-MG with LPS or α-Syn and followed their potential morphological alterations by real-time impedance measurements. These readings provide information on microglial morphological changes, which are continuously monitored for the whole period of LPS and α-Syn exposure. LPS is commonly used to induce activation of microglial or macrophage cells to mimic bacterial infections [25]. Impedance measurements were displayed as normalized cell index and indicated that LPS and α-Syn induced a continuous concentration-dependent increase in cell index, indicative of microglial activation. (Figure 4. a). Morphological changes, depicted as increased cell index were already detected within 120 min following exposure, and were continuously

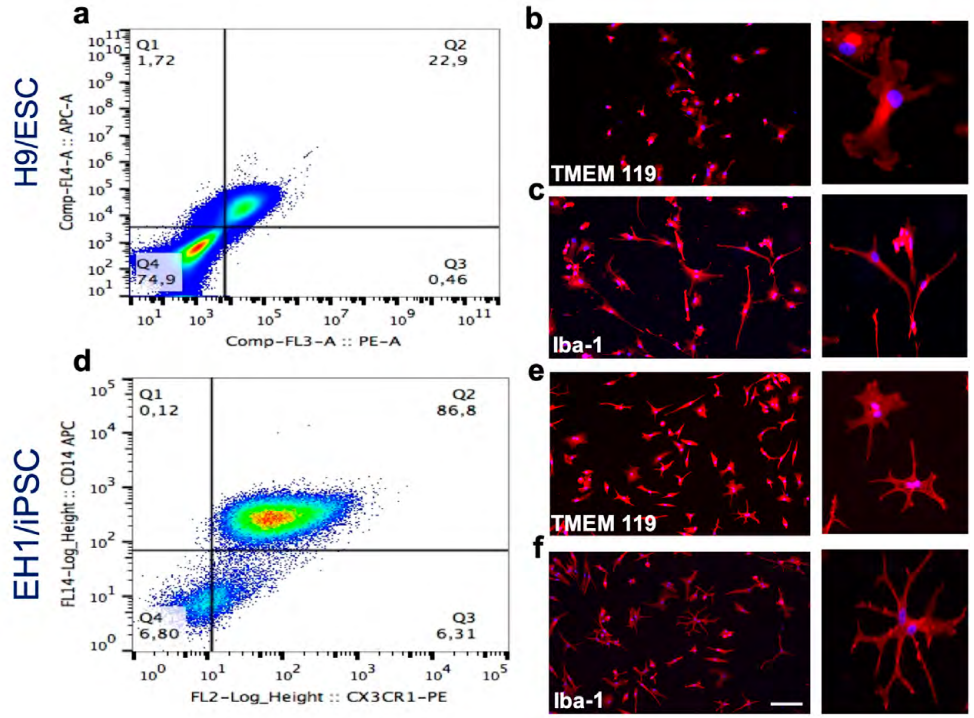


Figure 2. Characterization of iPSC-derived microglia from H9 and EH1 cells. Representative plot of the sorting gate used to isolate CD14+CX3CR1+ microglial progenitors via FACS between day 25 and 50 of differentiation from H9 **(a)** and EH1 cells **(d)**. A panel of representative images of microglia after immunofluorescent labeling for IBA1 and TMEM119 from H9 **(b-c)** and EH1 cells **(e-f).** Magnification 10x. Bar graph:10 µm

increasing during the following 24 hours. The increase in cell index reflected an increase in microglial shape, which indicated the time frame when microglia shifted its resting status into activation status. Microglial shape during activation changes from a small cell body with fine ramification into a larger amoeboid shape. These changes are easily detected by the cell impedance system. The impedance measurements were paralleled by immunofluorescence studies with a microglial marker (Iba-1 antibody) (Figure 4. b). Analysis of immunostainings of iPSC-MG challenged with LPS and α-Syn revealed comparable degree of morphological changes as primary microglia after exposure to LPS and α-Syn (Figure 4. c). Based on these findings, we proposed that the generated iPSC-MG were functionally active and resembled the morphology and activity of primary microglia.

Integration of iPSC-MG in brain organoids

Brain organoids are self-assembled three-dimensional cell aggregates generated from iPSC. The majority of generated brain organoids are populated by neurons and astrocytes and lack microglia since protocols to generate microglia were not readily available and the germinal origin of microglia is different than the one of neurons and astrocytes [26,27]. Brain organoids represent an excellent tool to model brain cell interaction and, therefore providing an

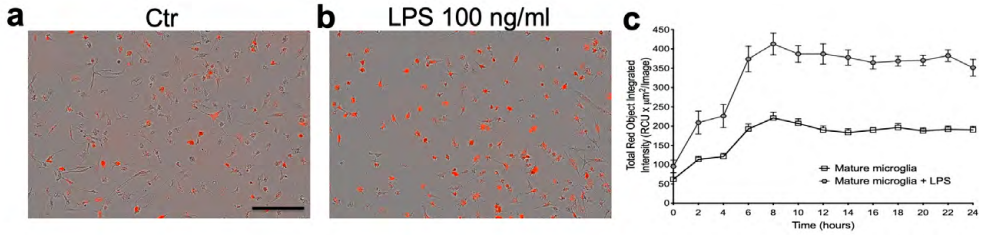


Figure 3. Phagocytic activity of human differentiated iPSC-MG. Cell imaging (Incucyte system) displaying phagocytosis reagent pHrodo red E. coli BioParticles being engulphed by cells without **(a)** and with LPS **(b)** with its corresponding time-lapse curves **(c)**. Magnification 10x. Bar graph: 300µm

exceptional opportunity to investigate human brain development and disorders. To study the ability of iPSC-MG to invade developing neural tissues and migrate within them, we developed organoids according to Lancaster et al., 2013 [21] and added mature iPSC-MG. The preformed 3D cortical organoids were cultured for 100 days *in vitro*.

To evaluate the composition of the cell population in the cerebral organoids at three months in culture, we investigated and detected the presence of general neural progenitor markers such as Nestin and KI-67 (Figure 5 a-b). Besides, immunolabelling assays revealed that cerebral organoids presented astrocyte markers, including GFAP and vimentin (Figure 5 c-d). At the same time, we identified neuronal nuclei (NeuN), B-III Tubulin and microtubule-associated protein 2 (MAP-2), components of neuronal signature (Figure 5 e-g) reflecting some maturity of the generated brain organoids. Under these conditions, we applied iPSC-MG to the organoid and observed an early integration of iPSC-MG first at the surface of the organoid and later in deeper layers of the organoid between day 3 to day 7 (figure 6.a-d). These data showed surviving iPSC-MG in those environments for at least nine days. After we fixated the organoids and we performed immunostaining with Iba-1, we detected a few positive cells inside the organoid (Figure 6.e-f).

Discussion

Modeling human diseases in rodents where microglia have been modified or ablated have been remarkably useful in demonstrating the beneficial and detrimental effects of microglia in the pathogenesis of a variety of neurodegenerative disorders. The main limitation to further our understanding of microglial biology and properly translate these data to a clinical setting has been the lack of an abundant source of normal and disease-specific human microglia. iPSC cells are a unique solution to obtain microglia due to unlimited self–renewal, and differentiation into any adult cell type [28]. The ability to produce unlimited amounts of functional microglia from people with different diseases may provide new tools for fundamental research in disease pathogenesis and drug development and at the same time unique therapeutical opportunities for neurodegenerative diseases as Parkinson or Alzheimer.

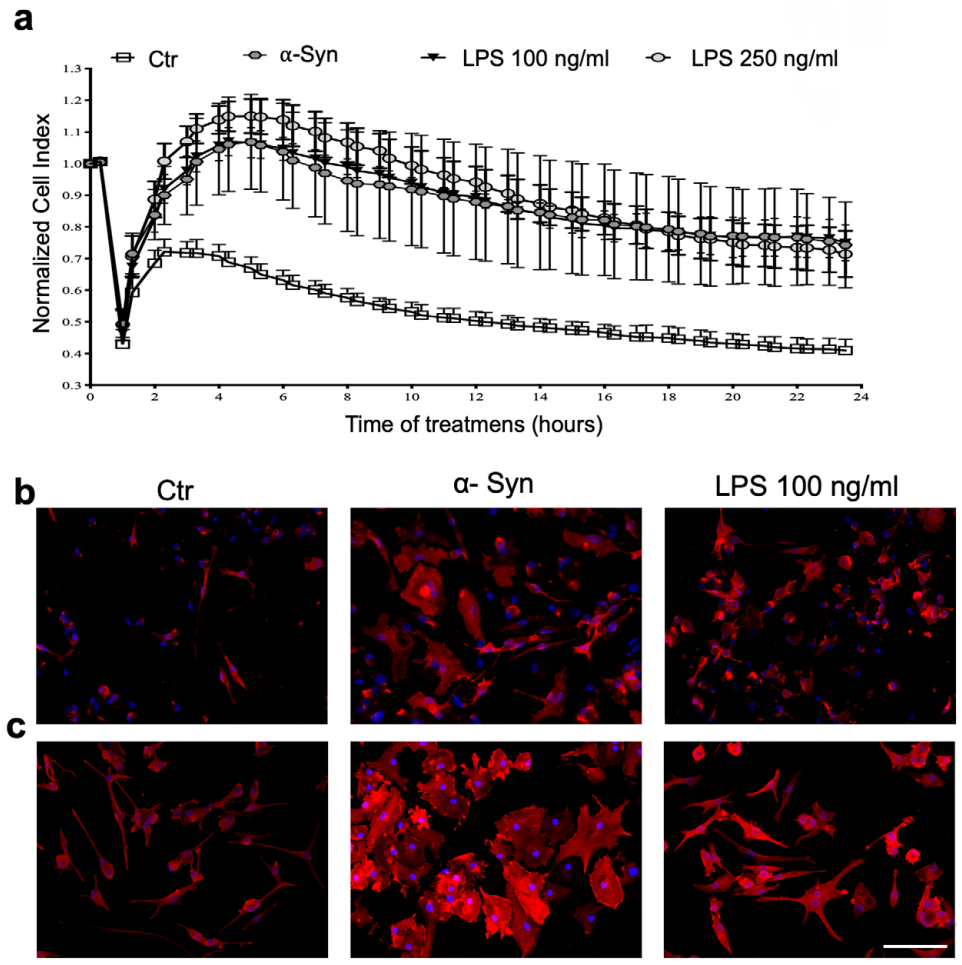


Figure 4. LPS and α-Syn induce iPSC-MG activation. (**a)** iPSC-MG cells were seeded in 96-well E-plates at a density of 15,000 cells/well and monitored with a real-time impedance-based xCELLigence system. After 24 hours in culture, cells were challenged with different two concentrations of LPS (100 and 250 ng/ml) and α-Syn (200 ng/ml) for 24 hours more. **(b)** Morphological alterations of activated microglia were visualized by immunostaining with Iba-1 antibody in iPSC-MG and **(c)** mouse primary microglia. Magnification 20x. Bar graph: 5µm.

To understand the role of brain cell interactions on neurodegenerative diseases, first we generated iPSC-MG that would later be integrated into a matured cerebral organoid. The strategy of iPSC-MG differentiation followed Douvaras and colleagues' protocol. *In vitro* hematopoietic differentiation of iPSCs resembled *in vivo* primitive hematopoiesis, where iPSC-derived myeloid progenitors would relate to *in vivo* primitive yolk sac myeloid progenitors [14]. Stimulating PSCs with a myeloid inductive medium followed by treatment with microglia-promoting medium and factors generated $KDR^{+}CD235a^{+}$ primitive hemangioblasts, which subsequently transitioned from $CD45^{+}CX3CR1^{-}$ to $CD45^{+}CX3CR1^{+}$ microglial progenitors *in vitro*. The maturation of microglial progenitors was achieved in the

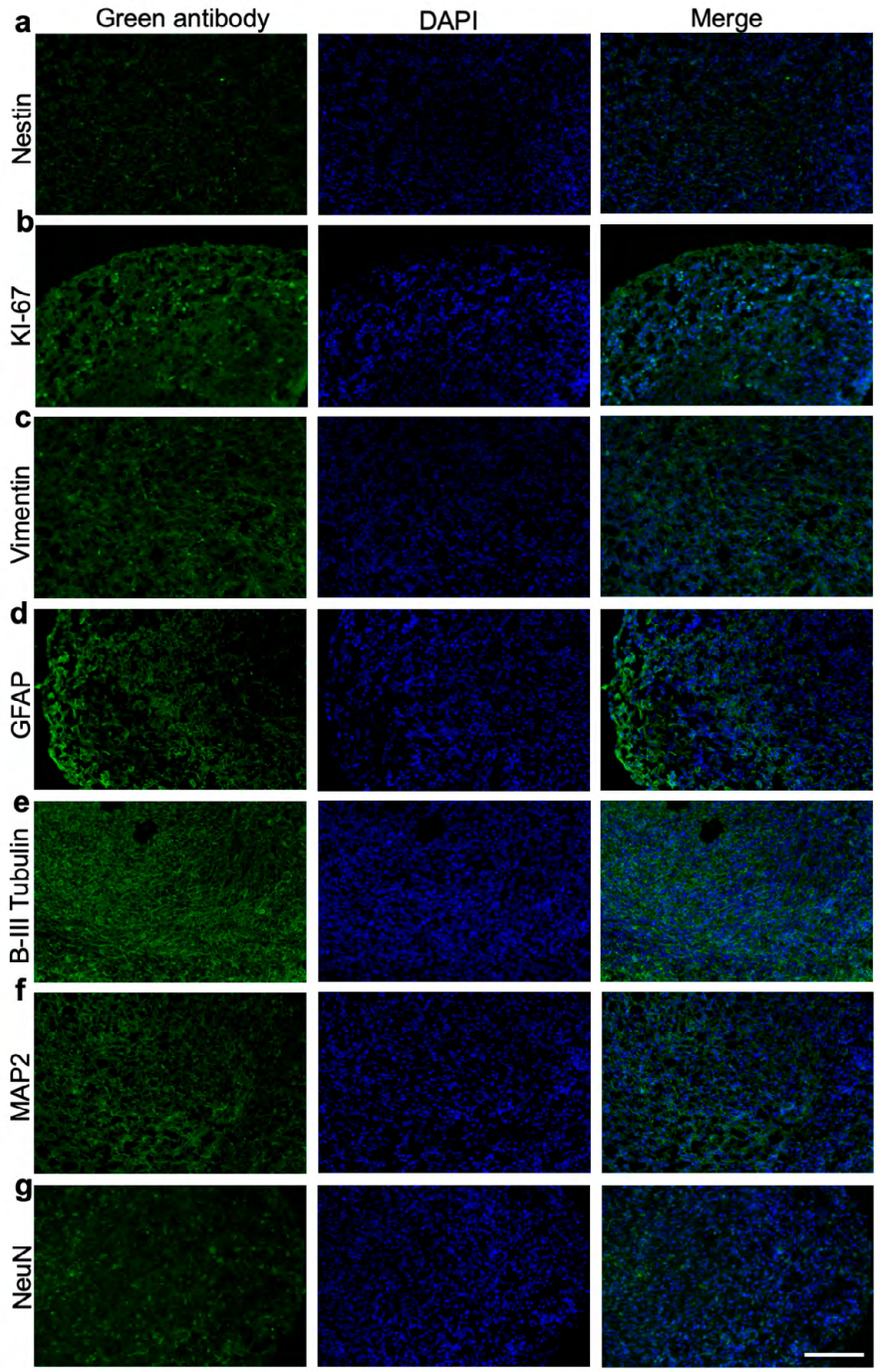


Figure 5. Generation and characterization of organoids according to Lancaster et al., 2017 Main markers in organoids of three months old with DAPI for Nestin (a), KI-67 (b), Vimentin (c), GFAP (d), B-III Tubulin (e), MAP-2 (f) and NeuN (g).

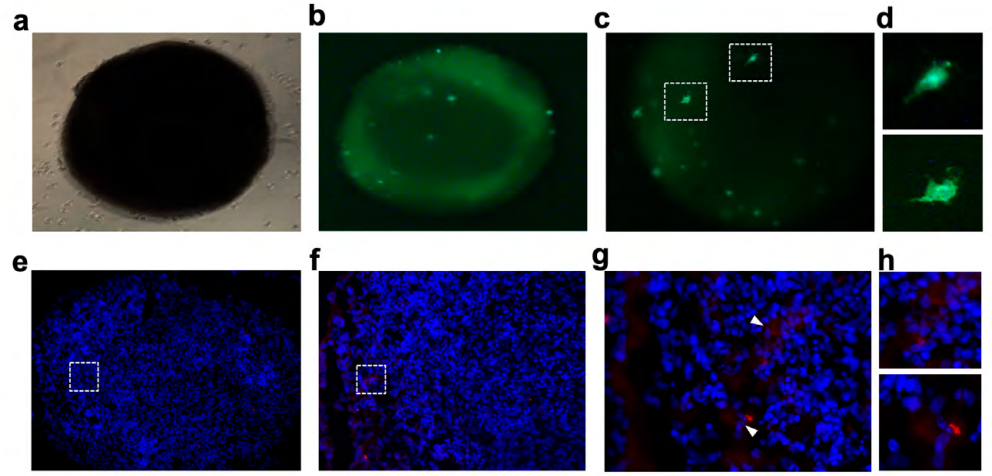


Figure 6. Microglia migrate into preformed cortical organoids. **(a)** Representative bright-field image of an organoid in the presence of iPSC-MG. Acquisition of the images were performed after two **(b)** and five days **(c-d)**. Immunostaining for Iba-1 (Red) in organoids after ten days in coculture of iPSC-MG with the organoids, objective 10x **(e),** 20x **(f)** and 40x **(g-h)**.

presence of GM-CSF and IL-34. GM-CSF is a growth factor that induces the proliferation and maturation of myeloid progenitors, giving rise to neutrophils, monocytes and macrophages, and eosinophils [29]. Likewise, IL-34 is a cytokine that IL-34 can binds to CSF-1R and stimulates the differentiation, proliferation and survival of monocytes, macrophages and osteoclasts and plays an important role in the development and maintenance of microglia [30,31].

To corroborate the reliability of the protocol, we evaluated an ESC and iPSC line, where we observed a striking difference between the production of microglia progenitors, 23% compared to 87%, respectively. Our data are in concordance with Douvaras et al., 2017 where they obtained a 40% to 60% efficiency in ESC and 45-95% from iPSC. Generation of microglia from iPSC derived from individuals with varying disease status, age, and sex, together with different reprogramming strategies account for the variability of yield per iPSC lines [13,14,32].

Phagocytosis is an essential function of microglia to mediate clearance of apoptotic cells, cell debris, extracellular protein aggregates, mainly during inflammatory processes triggered by injuries or neurodegenerative diseases [33,34]. Here, we demonstrated the functionality of generated iPSC-MG in a phagocytosis assay showing how microglia was able to phagocyte *E. coli* Bioparticles. The phagocytic capacity was further increased after addition of LPS, providing a similar answer as human microglia in a previously described study [35]. At the same time, immunofluorescence analysis with Iba-1 showed that LPS and α-Syn induced enlarged microglial cell bodies, indicative of an activation profile of iPSC-MG. These findings confirm the functional state of the microglia that we generated in concordance with differentiated microglial activity produced via different strategies [15,17,36].

We obtained organoids populated with different brain cells, such as neurons and astrocytes, and we added iPSC-MG to be able to study the interaction between neurons and microglia and to provoke an inflammatory answer. Analyzing the integration of iPSC-MG into cerebral organoid, we detected iPSC-MG in the organoid during the first days of co-culturing, while following ten days in culture, this quantity of microglia was reduced, maybe due to lack of growth factors inside the organoids. In conclusion, we successfully generated functional human microglia from iPSC that could be integrated into a cerebral organoid. The next logical steps include additional studies to obtain a better integration of microglia within organoids that would provide a suitable neuroglial environment to study cellular mechanisms of brain diseases.

Acknowledgments

The authors thank the group of Molecular pharmacology; group of Biomedical Sciences of Cells & Systems, Molecular Neurobiology, Faculty of Medical Sciences and Research Institute for the Biology of Ageing (ERIBA) of University of Groningen. Scholarship Colciencias call 647 (AMS-G) and program Abel Tasman from University of Groningen. A.M.D. is the recipient of a Rosalind Franklin Fellowship co-funded by the European Union and the University of Groningen.

Generation and functionality of human microglia-differentiated induced pluripotent stem cells

Supplemental figures

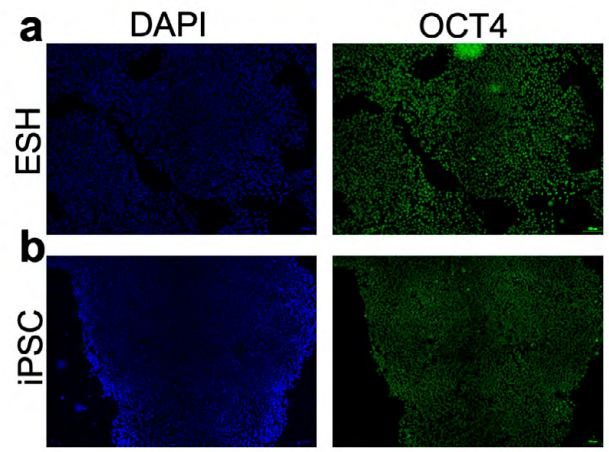


Figure supplementary 1. Immunoreactivity of OCT 4 (Green) in ESC and iPSC and cell viability with DAPI (Blue). Magnification 10x. Bar graph: 100 µm

References

[1] K. Kierdorf, D. Erny, T. Goldmann, V. Sander, C. Schulz, E.G. Perdiguero, P. Wieghofer, A. Heinrich, P. Riemke, C. Hölscher, others, Microglia emerge from erythromyeloid precursors via Pu. 1-and Irf8-dependent pathways, Nat. Neurosci. 16 (2013) 273–283. doi:doi:10.1038/nn.3318.

[2] H. Kettenmann, F. Kirchhoff, A. Verkhratsky, Microglia: New Roles for the Synaptic Stripper, Neuron. 77 (2013) 10–18. doi:10.1016/j.neuron.2012.12.023.

[3] M. Prinz, J. Priller, Microglia and brain macrophages in the molecular age: From origin to neuropsychiatric disease, Nat. Rev. Neurosci. 15 (2014) 300–312. doi:10.1038/nrn3722.

[4] M.L. Bennett, F.C. Bennett, S.A. Liddelow, B. Ajami, J.L. Zamanian, N.B. Fernhoff, S.B. Mulinyawe, C.J. Bohlen, A. Adil, A. Tucker, I.L. Weissman, E.F. Chang, G. Li, G.A. Grant, M.G. Hayden Gephart, B.A. Barres, New tools for studying microglia in the mouse and human CNS., Proc. Natl. Acad. Sci. U. S. A. 113 (2016) E1738–E1746. doi:10.1073/pnas.1525528113.

[5] R.M. Ransohoff, V.H. Perry, Microglial Physiology: Unique Stimuli, Specialized Responses, Annu. Rev. Immunol. 27 (2009) 119–145. doi:10.1146/annurev.immunol.021908.132528.

[6] A. Nimmerjahn, F. Kirchhoff, F. Helmchen, Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo, Science (80). 308 (2005) 1314–1318. doi:10.1126/science.1110647.

[7] F. Ginhoux, M. Greter, M. Leboeuf, S. Nandi, P. See, S. Gokhan, M.F. Mehler, S.J. Conway, L.G. Ng, E.R. Stanley, I.M. Samokhvalov, M. Merad, Microglia Derive from Primitive Macrophages, Science (80-.). 701 (2010) 841–845. doi:10.1126/science.1194637.

[8] U.-K. Hanisch, H. Kettenmann, Microglia: active sensor and versatile effector cells in the normal and pathologic brain, Nat. Neurosci. 10 (2007) 1387.

[9] J. Kershman, Genesis of microglia in the human brain, Arch. Neurol. Psychiatry. 41 (1939) 24–50.

[10] A. Verkhratsky, A. Butt, Microglia, in: W. Blackwell (Ed.), Glial Physiol. Pathophysiol., 2013: pp. 343–380. doi:10.1002/9781118402061.

[11] K. Askew, K. Li, A. Olmos-Alonso, F. Garcia-Moreno, Y. Liang, P. Richardson, T. Tipton, M.A. Chapman, K. Riecken, S. Beccari, A. Sierra, Z. Molnár, M.S. Cragg, O. Garaschuk, V.H. Perry, D. Gomez-Nicola, Coupled Proliferation and Apoptosis Maintain the Rapid Turnover of Microglia in the Adult Brain, Cell Rep. 18 (2017) 391–405. doi:10.1016/j.celrep.2016.12.041.

[12] E.A. Kimbrel, R. Lanza, Current status of pluripotent stem cells: moving the first therapies to the clinic, Nat. Rev. Drug Discov. 14 (2015) 681.

[13] E.M. Abud, R.N. Ramirez, E.S. Martinez, M.J. Carson, W.W. Poon, M. Blurton-jones, E.M. Abud, R.N. Ramirez, E.S. Martinez, L.M. Healy, C.H.H. Nguyen, S.A. Newman, iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases, Neuron. 94 (2017) 278-293.e9. doi:10.1016/j.neuron.2017.03.042.

[14] P. Douvaras, B. Sun, M. Wang, I. Kruglikov, G. Lallos, M. Zimmer, C. Terrenoire, B. Zhang, S. Gandy, E. Schadt, D.O. Freytes, S. Noggle, V. Fossati, Directed Differentiation of Human Pluripotent Stem Cells to Microglia, Stem Cell Reports. 8 (2017) 1516–1524. doi:10.1016/j.stemcr.2017.04.023.

[15] J. Muffat, Y. Li, B. Yuan, M. Mitalipova, A. Omer, S. Corcoran, G. Bakiasi, L.-H. Tsai, P. Aubourg, R.M. Ransohoff, others, Efficient derivation of microglia-like cells from human pluripotent stem cells, Nat. Med. 22 (2016) 1358.

[16] W. Haenseler, S.N. Sansom, J. Buchrieser, S.E. Newey, C.S. Moore, F.J. Nicholls, S. Chintawar, C. Schnell, J.P. Antel, N.D. Allen, M.Z. Cader, R. Wade-martins, W.S. James, S.A. Cowley, A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response, Stem Cell Reports. 8 (2017) 1727–1742. doi:10.1016/j.stemcr.2017.05.017.

[17] H. Pandya, M.J. Shen, D.M. Ichikawa, A.B. Sedlock, Y. Choi, K.R. Johnson, G. Kim, M.A. Brown, A.G. Elkahloun, D. Maric, others, Differentiation of human and murine induced pluripotent stem cells to microglia-like cells, Nat. Neurosci. 20 (2017) 753.

[18] P.W. Brownjohn, J. Smith, R. Solanki, E. Lohmann, H. Houlden, J. Hardy, S. Dietmann, F.J. Livesey, Functional Studies of Missense TREM2 Mutations in Human Stem Cell-Derived Microglia, Stem Cell Reports. 10 (2018) 1294–1307. doi:10.1016/j.stemcr.2018.03.003.

[19] J. Rustenhoven, T.I.H. Park, P. Schweder, J. Scotter, J. Correia, A.M. Smith, H.M. Gibbons, R.L. Oldfield, P.S. Bergin, E.W. Mee, others, Isolation of highly enriched primary human microglia for functional studies, Sci. Rep. 6 (2016) 1–11. doi:10.1038/srep19371.

Generation and functionality of human microglia-differentiated induced pluripotent stem cells

[20] S. Diemert, A.M. Dolga, S. Tobaben, J. Grohm, S. Pfeifer, E. Oexler, C. Culmsee, Impedance measurement for real time detection of neuronal cell death, J. Neurosci. Methods. 203 (2012) 69–77.

[21] M.A. Lancaster, M. Renner, C.-A. Martin, D. Wenzel, L.S. Bicknell, M.E. Hurles, T. Homfray, J.M. Penninger, A.P. Jackson, J.A. Knoblich, Cerebral organoids model human brain development and microcephaly., Nature. 501 (2013) 373–379. doi:10.1038/nature12517.

[22] D. Green, T.H. Oguin, J. Martinez, The clearance of dying cells: table for two, Cell Death Differ. 23 (2016) 915–926. doi:10.1038/cdd.2015.172.

[23] E. Janda, L. Boi, A.R. Carta, Microglial Phagocytosis and Its Regulation: A Therapeutic Target in Parkinson's Disease?, Front. Mol. Neurosci. 11 (2018) 1–8. doi:10.3389/fnmol.2018.00144.

[24] R. Nau, S. Ribes, M. Djukic, H. Eiffert, Strategies to increase the activity of microglia as efficient protectors of the brain against infections, Front. Cell. Neurosci. 8 (2014) 1–13. doi:10.3389/fncel.2014.00138.

[25] A.M. Dolga, T. Letsche, M. Gold, N. Doti, M. Bacher, N. Chiamvimonvat, R. Dodel, C. Culmsee, Activation of KCNN3/SK3/KCa2.3 Channels Attenuates Enhanced Calcium Influx and Inflammatory Cytokine Production in Activated Microglia, Glia. 60 (2012) 2050–2064. doi:10.1002/glia.22419.

[26] C. Schulz, E. Gomez Perdiguero, L. Chorro, H. Szabo-Rogers, N. Cagnard, K. Kierdorf, M. Prinz, B. Wu, S.E.W. Jacobsen, J.W. Pollard, J. Frampton, K.J. Liu, F. Geissmann, A lineage of myeloid cells independent of Myb and hematopoietic stem cells., Science. 336 (2012) 86–90. doi:10.1126/science.1219179.

[27] Q. Li, X. Han, J. Wang, Organotypic hippocampal slices as models for stroke and traumatic brain injury, Mol. Neurobiol. 53 (2016) 4226–4237. doi:10.1007/s12035-015-9362-4.

[28] S.P. Medvedev, A.. Shevchenko, S.M. Zakian, Induced Pluripotent Stem Cells: Problems and Advantages when Applying them in Regenerative Medicine, Acta Naturae. 2 (2010) 18–27.

[29] S.M. Liva, M.A. Kahn, J.M. Dopp, J. De Vellis, Signal transduction pathways induced by GM-CSF in microglia: Significance in the control of proliferation
, Glia. 26 (1999) 344–352. doi:10.1002/(SICI)1098-1136(199906)26:4<344::AID-GLIA8>3.0.CO;2-L.

[30] D.G. Walker, T.M. Tang, L.F. Lue, Studies on colony stimulating factor receptor-1 and ligands colony stimulating factor-1 and interleukin-34 in Alzheimer's disease brains and human microglia, Front. Aging Neurosci. 9 (2017) 1–15. doi:10.3389/fnagi.2017.00244.

[31] S. Boulakirba, A. Pfeifer, R. Mhaidly, S. Obba, M. Goulard, T. Schmitt, P. Chaintreuil, A. Calleja, N. Furstoss, F. Orange, S. Lacas-Gervais, L. Boyer, S. Marchetti, E. Verhoeyen, F. Luciano, G. Robert, P. Auberger, A. Jacquel, IL-34 and CSF-1 display an equivalent macrophage differentiation ability but a different polarization potential, Sci. Rep. 8 (2018) 1–11. doi:10.1038/s41598-017-18433-4.

[32] B. Popp, M. Krumbiegel, J. Grosch, A. Sommer, S. Uebe, Z. Kohl, S. Plötz, M. Farrell, U. Trautmann, C. Kraus, A.B. Ekici, R. Asadollahi, M. Regensburger, K. Günther, A. Rauch, F. Edenhofer, J. Winkler, B. Winner, A. Reis, Need for high-resolution Genetic Analysis in iPSC: Results and Lessons from the ForIPS Consortium, Sci. Rep. 8 (2018) 1–14. doi:10.1038/s41598-018-35506-0.

[33] H. Lian, E. Roy, H. Zheng, Microglial Phagocytosis Assay, Bio Protoc. 6 (2016) 1–12. doi:10.21769/BioProtoc.1988.

[34] R. Fu, Q. Shen, P. Xu, J.J. Luo, Y. Tang, Phagocytosis of microglia in the central nervous system diseases, Mol. Neurobiol. 49 (2014) 1422–1434. doi:10.1007/s12035-013-8620-6.

[35] E. Furube, S. Kawai, H. Inagaki, S. Takagi, S. Miyata, Brain Region-dependent Heterogeneity and Dose-dependent Difference in Transient Microglia Population Increase during Lipopolysaccharide-induced Inflammation, Sci. Rep. 8 (2018) 1–15. doi:10.1038/s41598-018-20643-3.

[36] R. Timmerman, S.M. Burm, J.J. Bajramovic, An Overview of in vitro Methods to Study Microglia, Front. Cell. Neurosci. 12 (2018) 1–12. doi:10.3389/fncel.2018.00242.

CHAPTER 9

General discussion, conclusions and perspectives

The understanding of the molecular mechanisms of AD and stroke together with the urgency to find new therapies are closely linked with the high impact that this research could have in society. Neurodegenerative disease is a general term for a range of conditions which mainly affect neurons in the human brain. The prevalence of neurodegenerative disorders is increasing, owing in part to extensions in lifespan [1]. Nowadays, there is no cure for any of these diseases, so our goal in this thesis was to shed more light on the molecular mechanisms of these diseases by focusing on a lipidomic approach and evaluate a potential protection of a naturally occurring terpene alcohol, namely linalool, in some of the most common neurodegenerative conditions such as AD and ischemia.

Since ancient times, humanity has used medicinal plant preparations for the treatment of its diseases, and, as research has evolved, new technologies have enabled more studies on natural compounds which derive from plants [2]. The health benefits reported by other authors [3] and preliminary studies in our group were the reason to choose different approaches to evaluate the protection of the naturally occurring monoterpene linalool. We described in two *in vitro* models for oxidative stress associated to neurodegenerative conditions (HT-22 cell line & primary neurons and astrocytes), one *ex-vivo* model for excitotoxicity associated to neurodegenerative conditions (OHSC) and two *in vivo* models for AD and ischemia (3xTg-AD mice & 2-VO in rats) the protection of the monoterpene linalool (Figure 1).

Linalool is susceptible to chemical modifications (oxidation, glycosylation, esterification, and methylation) due to the two double bonds and the hydroxyl group (OH) in its structure [4,5]. Linalool exhibits chiral properties due to the OH group in the third carbon (C3), and it has two enantiomers and the racemic (rac) form. The pharmacological differences between linalool enantiomers have been shown on physiological parameters on human stress reactions assessing endocrine and autonomous nervous system [6] and in sedative effect in humans [7]. The chiral influence of other optically active monoterpenes on behavior experimental tests in mice also was confirmed [8]. Interestingly, Sousa et al., 2010 showed that both enantiomers and rac-linalool have similar anticonvulsant activity, but differ in their potencies where rac form was the most potent [9].

Linalool is neither phototoxic [10] nor genotoxic [10,11]. The evaluation of linalool *in vitro* in bacterial test systems (Ames assay) and *in vivo* in mammalian test systems (micronucleus assay) showed no evidence of mutagenic or genotoxicity [12]. Nevertheless, the toxicity was evaluated in Sprague–Dawley rats with the maternal NOAEL (No observed adverse effect level) and the developmental NOAEL were 500 mg/Kg bw/day and 1000 mg/Kg bw/day, respectively [12]. Besides, after oral exposure in rats, NOAEL value was 500 mg/Kg bw/day and the margin of safety was major to 5000 [13]. These concentrations are much higher compared with 25 mg/kg that we and other colleagues have used in models of AD, ischemia or other disease models [14–17].

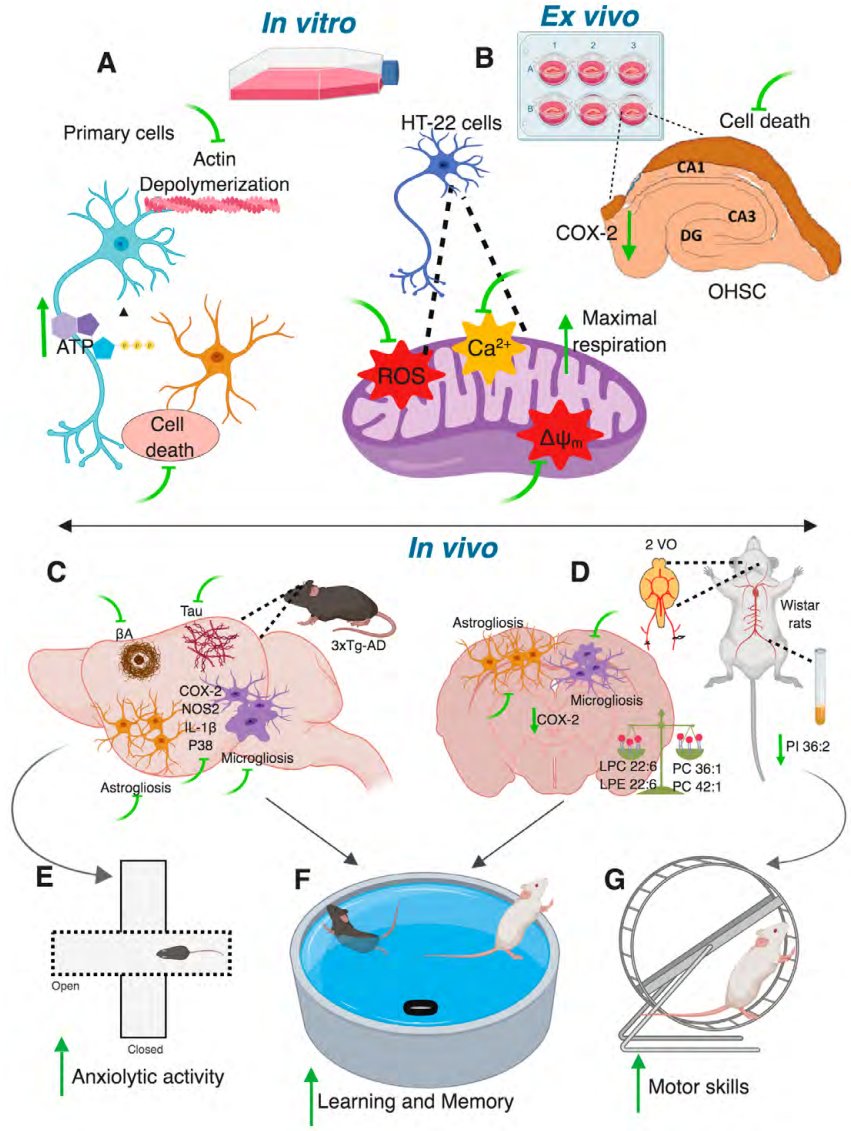


Figure 1. Protection by linalool in different models of neurodegenerative diseases. Green arrows represent the linalool effect on the therapeutic targets **A.** Primary and HT-22 cells protection by linalool: neurons and astrocytes in culture were tested with excitotoxicity by glutamate and linalool we showed linalool recovered the cell viability and levels of ATP; decrease the actin depolymerization and cell death by linalool; In HT-22 cells linalool reduced ROS, calcium mitochondrial, recovered mitochondrial membrane potential and increased maximal respiration in mitochondria. **B.** We observed in OHSC that linalool reduced cell death mainly in CA1 from the hippocampus and COX-2 expression after NMDA toxicity. **C.** Oral administration of the linalool ameliorated β-amyloidosis, tauopathy, microgliosis and astrogliosis through regulation of proinflammatory markers as COX-2, NOS2, IL-1B, P38MAPK in an aged triple transgenic AD model mouse. **D.** Linalool reduced astrogliosis and microgliosis in the hippocampus modulating COX-2 protein of rats subjected to 2 vessels occlusion (global cerebral ischemia); we found linalool was able to maintain the homeostasis of lipids such as LPC 22:6, LPE 22:6, PC 36:1 and PC 42:1 in hippocampus and PI 36:2 in serum**. E-G.** Linalool reduced cognitive and emotional deficits in 3xTg-AD and sensorimotor and memory deficits in ischemic rats.

Experimental studies on rats, performed with 14 C-labelled linalool (500 mg/kg bw), indicated that it is quickly absorbed from the intestinal tract after oral or gavage administration. After absorption, most of the linalool is quickly metabolized in the liver to polar compounds that are mainly excreted in the urine as free form or conjugates. As an example, Aprotosoaie et al, 2014 detected 8-Hydroxy- and 8-carboxy-linalool as significant metabolites following 20 days administration of 800 mg linalool/kg bw/day [18] or dihydro-, tetrahydrolinalool identified in rodent urine. Besides, a high proportion of oral linalool follows intermediary metabolism pathways and is eliminated in exhaled air as CO_2 [11].

We demonstrated the protection of linalool *in vitro* in a model of 1) excitotoxicity induced by glutamate in neurons and 2) of oxytosis in neuronal HT-22 cells. HT-22 cells lack NMDAR; therefore glutamate stimulation initiates a distinct pathway of cell death independent of NMDA-related increased neuronal excitability. Our data are supported by previous *in vitro* studies in which PC12 were challenged by glucose/serum deprivation (GSD) conditions, and linalool was able to prevent the cell death following 8 hours of GSD [19]. In parallel, Park et al., described neuroprotective effects of linalool against oxygen glucose deprivation/ reoxygenation (OGD/R) that induced cortical neuronal injury. This study showed that linalool significantly reduced intracellular oxidative stress, scavenged peroxy radicals, and decreased the activities of SOD (superoxide dismutase) and catalase [20]. *In vitro* screening of linalool's antioxidant potency depicted moderate superoxide scavenging and negligible H_2O_2 scavenging activity [21], in contrast to the protective effects against H_2O_2 induced oxidative stress in brain tissue [22].

Mitochondria play an essential role in cellular metabolism, where oxygen consumption through the electron transport system (ETS) is tightly coupled to ATP production and regulate metabolic demands. Mitochondrial respiration take part in key processes such as oxidative phosphorylation, ion gradients, membrane potential, ROS generation and heat dissipation [23,24]. We demonstrated that linalool *in vitro* restored intracellular ATP levels in a glutamate excitotoxicity model suggesting that mitochondria play an essential role in the linalool-mediated protection. In consequence, we conducted high-resolution respirometry experiments to evaluate mitochondrial metabolic functions. We observed an increase in maximal oxygen consumption in response to linalool. Maximal oxygen consumption rate attained by adding the uncoupler FCCP mimics a physiological "energy demand" by stimulating the respiratory chain to operate at maximum capacity, which causes rapid oxidation of substrates (sugars, fats, amino acids) to meet this metabolic challenge [25]. Our data indicate that increased maximal respiration and spare capacity in response to linalool is associated with increased metabolic fitness allowing the cell to cope better with stress.

On the other hand, linalool was able to recover the homeostasis of phospholipids such as PC in hippocampus and PI in serum in rats one-month postischemia. It has been described that linalool reduced cellular lipid accumulation regulating PPARα-responsive genes and

significantly induced FA oxidation, and its effects were markedly attenuated by silencing PPARα expression. In mice, the oral administration of linalool for three weeks reduced plasma TG concentrations in Western-diet-fed C57BL/6J mice [26]. Linalool reduces the expression of 3-hydroxy-3-methylglutaryl CoA reductase via sterol regulatory element binding protein-2- and ubiquitin-dependent mechanisms [27] and inhibits proliferation and cholesterogenesis in liver-derived (HepG2) and extrahepatic (A549) cell lines [28]. Besides, linalool could inhibit intracellular lipid in 3T3-L1 adipocytes, indicating that S-(þ)-linalool might play an essential role in controlling body weight [29].

Interestingly, our data showed that linalool-treated 3xTg-AD mice exhibited an increased frequency of entry into the open arms, increased head-dipping, reduced grooming and rearing frequencies compared with those of vehicle-treated 3xTg-AD mice. Our data confirm the results of previous studies reporting that inhaled linalool has anxiolytic properties, increased social interaction, and decreased aggressive behavior [30,31]. Chen et al., 2015 described that linalool 500mg/kg significantly increase the time that mice spent in the open central area detected by the open field test and also the time they spent in the open arms of the EPM. This concentration did not render any side effects on motor activity, indicating excellent anxiolytic responses [32]. However, other study did not report any difference in the number of entries to the open arms using 125 mg/kg linalool [33]. In our study, we observed significant improvements in the neurological score, motor function and cognitive function by linalool 25 mg/kg given orally in 2VO model of cerebral ischemia. In accordance with the data from 3xTg-AD, linalool treatment effectively decreased the escape latencies in the learning, retention and re-learning tests compared with control groups in both models. Recently, Xu et al. corroborated this effect in another model of AD, where 100 mg/kg linalool reduced the escape latencies, increased the escape rate, and increased the time spent exploring the platform location in MWM test [34].

Reduction of COX-2, astrogliosis and microgliosis were common processes in our studied models of AD and stroke. Therefore, our data is indicative of a potential anti-inflammatory effect of linalool in conditions of stress. Inflammatory reactions, caused by diseases such as diabetes and cardiovascular disease, can be alleviated by linalool, which interacts with proteins such as COX-2, NF-κB, and nuclear factor erythroid 2-related factor 2 (Nrf-2) [35]. Linalool, mainly (-)-linalool, may also interact with nitric oxide synthase (NOS) enzyme, inhibiting the production of nitric oxide (NO) without reducing the enzyme synthesis [36]. Moreover, Li et al. suggested that linalool inhibited LPS-induced inflammation in BV2 microglia cells, both via the NF-κB pathway and also by the activation of Nrf2/ heme oxygenase-1 (Nrf2/HO-1) signaling pathway [37].

We observed microglia activation in AD and stroke animal models, in concordance with morphological alterations reported in postmortem AD and stroke brain tissue [38–40] suggesting that innate neuroinflammatory processes play a role in the progression of

neurodegenerative diseases. Recent findings reported a molecular diversity of murine microglia on a temporal and spatial axis during ontogeny, homeostasis, adulthood, and aging [41–43]. iPSC technology offers the opportunity of culturing patient derived cells and in this way this technology represents a valuable tool for studying disease etiologies at molecular and cellular levels. Based on these observations, we have decided to generate microglia from healthy control subjects by using a modified protocol of Douvaras et al., 2017 [44]. Protocols to generate microglia are fairly recent, just few years ago, Muffat et al, 2016 [45] described the first protocol to produce iPSC-derived microglial-like cells, and nowadays there are around 10 protocols described in literature.

These reported protocols share similar patterns in the microglial differentiation steps, with one common denominator at the beginning of the protocol being represented by BMP4 and at the end during the maturation phase IL-34, MCSF and GM-CSF. BMP4 regulates proliferation and differentiation, cell-fate determination, inhibits neurogenesis and induces neural stem cell (NSCs) glial differentiation in the adult CNS [46,47]. During differentiation, these protocols are using a variety of growth factors and interleukins such as IL-3, IL-6, TPO, VEGF, and SCF. During the last steps of differentiation, IL-34 is added together with GM-CSF to promote the maturation of microglia. These substances are also required for the differentiation, proliferation and survival of monocytes, indicating the similarities between monocyte and microglia functionality [48,49].

The efficiency in the generation of microglia progenitors showed a marked difference between EHS and iPSC lines, 23% compared to 87%, respectively. Our data are in concordance with Douvaras et al., 2017 [44] where they obtained a 40% to 60% efficiency in ESC and 45-95% using iPSC. Likewise, it has been demonstrated that generation of microglia from iPSC derived from individuals with varying disease status, age, and sex, and the reprogramming strategies highly influence the variability of yield per iPSC lines [50,51].

Our review presents an overview of the role of microglia in human neurodegenerative diseases, and different protocols that can be employed for generation of microglia from iPSC. We discuss the variability between growth factors, medium, time scale for the production of microglia that affect the yield, scalability and purity of these cells. We demonstrate the purity of microglia cells by immunostaining with markers IBA-1 and TMEM119, similarly, other groups used these markers or CD11B, CD11C, P2RY12, CD45 [45,50,52–54]. TMEM 119 (Transmembrane protein 119) has been validated as a unique marker to differentiate microglia from related cell types as macrophages or monocytes [55].

In the same way, isolation by FACs is based on different antibodies according to the microglia progenitors. Abud et al.,2017 reported the isolation of hematopoietic progenitors after sorting with CD43 [50] and Douvaras et al.,2018 described that microglia progenitors were isolated with CD14 and CX3CR1 [44]. However, functional assays are reproducible with both protocols and they reported high purity, while transcriptomic analysis in almost all the protocols showed a unique signature for microglia with genes such as Ferls, C1qa, P2ry12, Pros1, Mertk and Gas6 [56]. Whole-transcriptome principal component analysis (PCA) highlights the differentiation trajectory of monocytes, macrophages and dendritic cells compared to microglia. In addition, the comparison between generated microglia and its potential resembling with neonatal microglia or adult microglia form patients was shown [50,57]. Nevertheless, Garcia et al, 2018, point out similarities between gene expression in iPSC macrophages and iPSC microglia and therefore refer to them as iPSC-microglial-like cells (iPSC-MGLCs) [58].

In our studies, functional assays demonstrated the maturity of human differentiated microglia, as showed by their response to a variety of stimuli and detected by xCELLigence real-time impedance measurements and phagocytosis assays. Nevertheless, ongoing experiments are realized to confirm the unique signature of microglia. Discovering the pertinent immune components may lead to the development of new therapeutic targets to decelerate or even cease neurodegenerative diseases.

Lipid profile in neurodegenerative diseases

The field of lipidomics has recently converged into the neuroscience field trying to elucidate the role of lipids, to characterize their biologic role, and their relationships concerning the expression of proteins involved in lipid metabolism and function in aging and neurodegenerative diseases [59]. The composition of brain lipids in the cerebral cortex is altered with age and brain injuries [60]. In cerebral ischemia and AD processes, important changes have been determined in the composition of fatty acids, cholesterol, phospholipids, and sphingolipids. These alterations can produce other intermediates that in turn can change their overall lipid homeostasis. These processes highly depend on the time elapsed post-ischemia, stages of AD and the brain region, where the composition of lipids regulate the molecular pathways of cell death.

We evaluated the potential alteration of the hippocampal and peripheral phospholipid profile in long-term postischemia associated with cognitive impairment in rats. The main changes on phospholipids (PLs) were associated with hippocampal dysfunction, such as the decrease in phosphatidylcholine (PC 32:0, PC 34:2) and the increases in PLs dysfunction in membrane structure and signaling. In particular, lysophosphatidylethanolamine (LPE 18:1) and phosphatidylserine (PS 38:4), in the hippocampus were associated with these effects. Complementarily, PC 34:2, and ether-PC 34:1 decreased, while Lyso-PC 20:4 and phosphatidylinositol (PI) 36:2, as neurovascular state sensors, increased in the serum, as we can observe in Figure 2. Interactions between phospholipids and other lipid mediators

are involved in maintaining lipid asymmetry, a dynamic process, which they are required for maintaining neural membrane functions such as neuroplasticity and vesicular transport [61]; regulation of these functions after ischemia is fundamental to understand the pathophysiology of CVD.

Similarly, we showed differential lipid patterns between pure vascular dementia as CADASIL, sporadic Alzheimer disease (SAD) and control patients. The lipid patterns were investigated in gray matter (GM), white matter (WM) and cerebrospinal fluid (CSF). PLS-DA analysis discriminated species with the higher sMC index, such as PS 44:7 (18:1/22:6)/ /LPE 18:2 in GM; PE 32:2 (16:1/16:1)/PC 44:6 (18:0/22:6) in WM, and ePE 38:2 (18:0/20:2)/ePC 34:3 (14:0/20:3) in CSF. These findings were supported by high correlation profiles of PLs between both pathologies as indicated in the heat maps. Interestingly, common sub-phospholipidic species discrimination was obtained in both types of dementia in respect to the control, such as, PS 44:7/LPC 22:5 in GM, highlighting a transversal phenomenon that impact the brain parenchyma in a similar manner. Moreover, a comparable immunoreactivity pattern supported these results by neural (astrogliosis, microgliosis, dendrite loss) and vessel population (retraction and extravasation) markers in both types of dementia.

Phospholipids are biologically relevant molecules, which form cell membranes and are

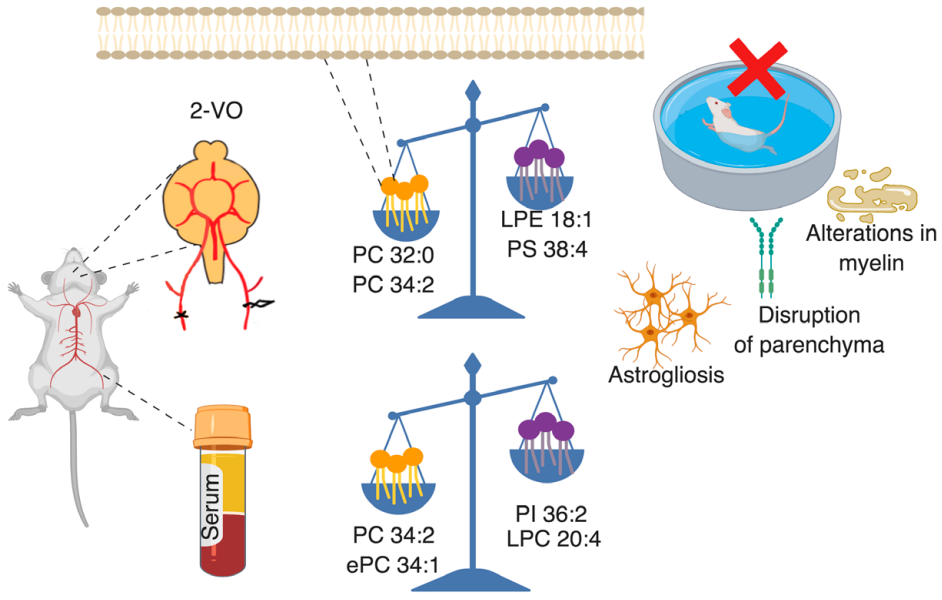


Figure 2. Regulation of phospholipids after one-month post-ischemia. Phospholipids were evaluated after one month of 2 vessel occlusion model in rats (multifocal injuries) in the hippocampus and serum. We detected a reduction of PC 32:0, PC 34: 2 and increase of LPE 18:1 and PS 38:4 in the hippocampus. Meanwhile, in serum we observed an increase of PI 36:2 and LPC 20:4 and reduction of PC 34:2, and ePC 34:1. Besides we observed rats with ischemia has cognitive deficits and alterations in myelin, cerebral parenchyma, and astrogliosis. LPC, Lysophosphatidylcholine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; ePC, ether phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine.

involved in the behavior of membrane proteins, receptors, enzymes, and ion channels. They are involved in maintaining lipid asymmetry and vesicular transport [62]. Increased activity of phospholipases, increased anabolism of lysophospholipids, peroxisomal dysfunction, imbalances in the levels of saturated/unsaturated fatty acids and oxidative stress are associated with neurodegenerative diseases and are indicative of the progress of the pathology [63].

Phosphatidylcholine (PC) is the most abundant glycerophospholipid in the membrane and plays a crucial role in cellular signaling [64]. We observed an imbalance in the production of PC and LPC one-month post-ischemia while some species of PC were reduced and some species of LPC were increased. However, in CADASIL and SAD tissue we observed both forms of PC 42:4 and 44:6 were increased. Our results in ischemia are in concordance with previous studies where PC was reduced after 24 hours following the injury [65] and also in neurodegenerative diseases. In particular, a reduction of PC and PE levels was detected in the cerebral cortex of patients with AD, which is potentially linked to the roles of $PLA_{2,}$ PLD in BA activation [66,67]. In our study, we observed an increase of PC in SAD, while in a different study alterations in this PL in the frontal cortex, parietal, and temporal region of AD patients were not observed [68]. Based on these results, the PC distribution patterns depend on the fatty acid composition, which reflects the heterogeneous membrane lipid compositions in distinct cell types. In addition, PC species are composed of saturated palmitic (16:0) and stearic acid (18:0), monounsaturated oleic acid (18:1) and polyunsaturated linoleic acid (18:2). This composition of membrane lipids determines their fluidity, curvature, permeability and signaling pathways that can mediate proinflammatory cytokines release, and worsen the cellular homeostasis in neurological diseases. Our results confirmed the imbalance in PLs and LPLs after global cerebral ischemia, CADASIL and SAD.

Phosphatidylinositol (PI) was present in low percentages in the serum of ischemic rats. However, we observed a remarkable increase that could be used as a predictive marker in the time-course after cerebral ischemia. PI is characterized by the phosphorylation of the inositol head group of phosphoinositide, with a rapid and reversible phosphorylation rate, which critically participates in signal cascades, cytoskeletal remodeling, and intracellular membrane trafficking [69,70]. Similar studies showed stimulation of 3HInositol monophosphate formation via excitatory amino acids signaling that is greatly enhanced in hippocampal slices 24 h or 7 days after reperfusion in a four vessels occlusion-ischemia model [71]. Furthermore, PI increased in response to calcium stimuli in the brain [72], while calcium imbalance was associated with mitochondrial dysfunction [73], vascular alterations and has been recognized to promote a toxic environment in neurological diseases [74].

Phosphatidylserine (PS) plays a central role in the brain cell membrane fluidity, transmission of brain cell activity and neural information [75]. We observed PS 42:7 was the main

discriminant PL in GM where in CADASIL and SAD groups the levels of PL were reduced compared to the controls. While, Wood et al., 2015 reported that the levels of PS were unaltered in the CSF, GM or WM [76], other groups reported that PS levels gradually decrease with increasing of age, affecting memory and cognitive ability. In AD cases this reduction is higher compared with control subjects [77]. Besides, we observed PS 42:7 (18:1/22:6) was composed by oleic acid and DHA was altered in dementia groups, correlating with a lower plasma concentration. In addition, other fatty acids have been associated with cognitive decline in both healthy elderly people as well as patients with AD [78].

Lysophospholipids (LPLs) are proinflammatory phospholipids that are synthesized in the brain through the action of phospholipase 1 (PLA_1) and PLA_2 on PLs such as PC and PE, which are metabolized by lysophospholipases and acyltransferases. LPLs, such as LPC, not only directly interact with ion channels and neurotransmitter receptors but also indirectly modulate their activity and neural membrane fluidity [79]. LPC 20:4 and LPE 18:1 were increased in the ischemic group compared to the other groups. LPLs such as LPC and LPE alter the membrane permeability and disturb the osmotic equilibrium. On the other hand,

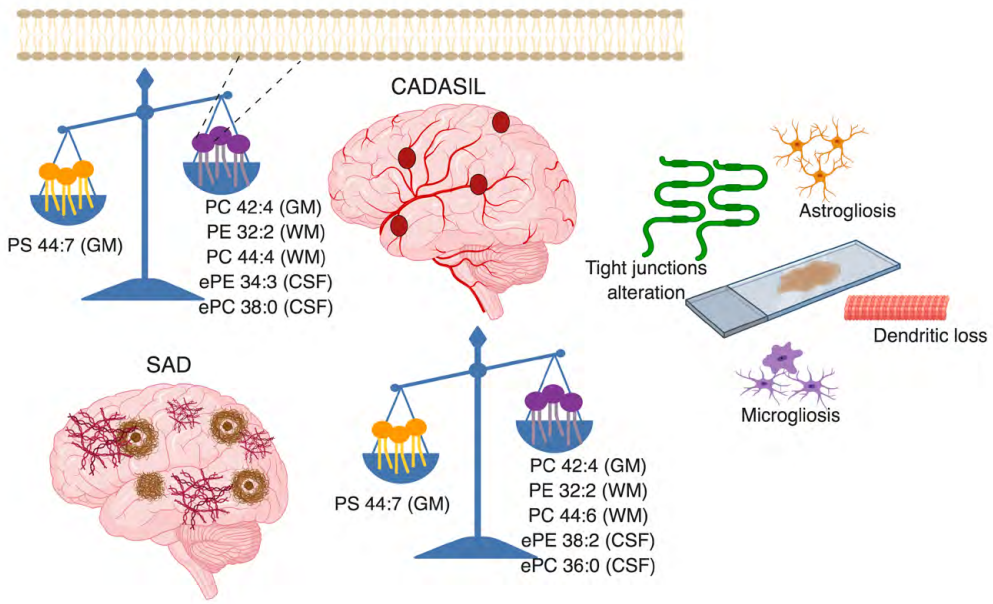


Figure 3. Alterations in lipid profile from dementia cases. We undertook lipidomics analysis of postmortem temporal cortex (GM), subjacent white matter (WM) and cerebrospinal fluid (CSF) to define potential biomarkers that distinguish cognitively intact subjects from CADASIL and sporadic Alzheimer disease (SAD). PS 44:7 was reduced in CADASIL and SAD groups in the GM. We observed in CADASIL an increase of PC 42:4 (GM); PE 32:2 and PC 44:4 (WM); ePE and ePC 38:0 (CSF). In SAD group we observed an increase in PC 42:4 (GM); PE 32:2 and PC 44:6 (WM); ePC 38:2 and ePC 36:0 in CSF. Besides, we shown alterations in astrocytes, microglia, tight junctions and dendritic loss in dementia groups after immunostaining. PC: phosphatidylcholine; ePC ether phosphatidylcholine; PE, phosphatidylethanolamine; ePE, ether phosphatidylethanolamine; PS, phosphatidylserine.

we observed an increase of ether-PC (ePC) in the CSF of CADASIL and SAD groups. Ether lipids represent one of the major structural component of cell membranes: their incorporation in PLs alters the membrane physical properties and affects membrane dynamics. This property is particularly important in higher order membrane structures such as those found in myelin, evidenced by its enrichment in plasmalogens [80].

PLs are multifunctional molecules, are the major constituents of membranes and are responsible for the membrane bilayer. Their physiological roles have been challenging to be identified and are likely to vary in different tissues, metabolic processes, and developmental stages. Our findings are potentially useful for improving prediction and intervention after cerebral ischemia, SAD and CADASIL and form the basis for a future understanding of PL dysfunction in neurological pathologies and will help to propose new biomarkers and therapeutic targets.

Main Conclusions

Oral administration of linalool at an advanced stage of AD in 3xTg-AD model mice reversed the histopathological hallmarks of AD (amyloidosis, tauopathy, astrogliosis, and microgliosis) and restored cognitive and emotional functions evaluated by analysis of Morris water and elevated plus maze behavioural tests, respectively.

- The treatment with linalool prevented glutamate-induced cell death of neurons and astrocytes, retraction of neural processes, actin cytoskeleton depolymerization and recovering ATP levels following glutamate challenge.
- Post-ischemic neurological, motor and cognitive impairments were prevented by the oral linalool treatment. Linalool reversed the phospholipid alterations, reduced astrogliosis and COX-2 expression in the hippocampus.
- Administration of linalool to HT-22 cells challenged by toxic concentrations of glutamate, reduced cell death, mitochondrial ROS, lipid peroxidation, calcium levels, and preserved mitochondrial membrane potential.
- Linalool increases maximal respiration and spare capacity in HT-22 cells contributing to its neuroprotective capacity.
- Linalool reduced NMDA-induced damage in the hippocampus and the increase of the gene expression of *COX-2* in organotypic hippocampal slices.
- Based on our findings, we propose that linalool may be a potential candidate for further preclinical studies and future translational studies on neurodegenerative diseases
- Phospholipids associated with neurotransmission, such as phosphatidylcholine (PC 32:0, PC 34:2, PC 36:3, PC 36:4, and PC 42:1) were reduced. In addition, increases in PLs involved in the membrane structure and signaling, such as lysophosphatidylethanolamine (LPE 18:1, 20:3, and 22:6) and phosphatidylserine (PS 38:4, 36:2, and 40:4), in the

hippocampus were detected one month post-ischemia

- PC (PC 34:2, PC 34:3, PC 38:5, and PC 36:5) and ether-PC (ePC 34:1, 34:2, 36:2, 38:2, and 38:3) were decreased, while Lyso-PC (LPC 18:0, 18:1, 20:4, 20:5, and LPC 22:6) and phosphatidylinositol (PI 36:2, 38:4, 38:5, and 40:5) increased in the serum of ischemic rats.
- PLs are a complex network involved in neurotransmission precursors, myelin composition and inflammation response in GM, WM and CSF respectively affecting dementias as CADASIL and SAD.
- We observed a reduction of PS 44:7 in both dementia groups in GM. In other regions we observed an increase in species of PC, PE, ePE and ePC. Although we reported phospholipid changes being not specific and sensitive for just one PL as a diagnostic biomarker, an overview of lipid profiles could give a prodromal biomarker of dementia.
- We successfully generated functional human microglia from iPSC that could be used to interrogate emerging questions associated to inflammatory processes in neurodegenerative diseases

Our findings demonstrated that administration of linalool reduce the main hallmarks in AD and in ischemic stroke, effects being associated with the improvement in motor, memory and cognitive skills. Although, more studies are necessary to properly understand its mechanism of action, we have demonstrated the linalool capacity against inflammatory processes, oxidative stress, and mitochondrial dysfunction. Linalool modulate the mitochondrial functions through the increase of maximal respiration, spare capacity and phospholipid regulation. These findings suggest that linalool may be a good candidate for further preclinical studies and future translational studies on neurodegenerative diseases.

In our lipidomic approach we detected interesting changes in phospholipids in ischemic stroke, AD and CADASIL in brain samples, CSF and serum that could help to unravel mechanism in these diseases and help to propose different targets based on the evaluation of phospholipid profile.

Perspectives

Therapy with natural products has shown great advances for the treatment of several diseases. These natural products can act mostly on a single target or on multiple targets, increasing in this way the benefit that could be produced in patients. The results obtained in this research propose linalool as an effective treatment for AD in a triple transgenic mouse at 21 months and one-month postischemia. It would be interesting to carry out a study to evaluate the preventive potential of this monoterpene in both models. Similarly, in the model of cerebral ischemia, the long-term effect of linalool could be evaluated, since it is known that sequelae of stroke can be observed up to 4 months.

The question of how linalool regulates the mitochondrial function and phospholipids remains to be solved. Therefore, future study involving genetic modification of mitochondria respiratory complexes and phospholipids such as PC and PI would give us insights to better understand the mechanism of action of this terpene.

Assessment of bioavailability, determination of pharmacokinetic characteristics and use of pharmacokinetic/pharmacodynamic modeling can aid in more rational use of linalool. This will allow to propose this molecule to begin phase 0 designed by the la Food and Drug Administration's (FDA) on Exploratory Investigational New Drug (IND) Studies. Phase 0 trials that include the administration of single subtherapeutic doses of the study drug to a small number of subjects (10 to 15) to gather preliminary data on the agent's pharmacokinetics (what the body does to the drugs). In this way we would be able to validate the therapeutic potential of linalool and allow in a future its pharmacological application in neurodegenerative diseases.

We showed a phospholipid profile in different diseases as SAD, CADASIL and stroke. A metabolomic profile including other lipids involved in brain damage, such as sphingolipids, gangliosides and cardiolipin, could help to understand the pathology and progression of these diseases.

We showed the generation of functional microglia from iPSC and next studies should include comparison of biological activity of different lines of patients with neurodegenerative diseases such as AD and PD. Integration in a 3D spheroids would provide a suitable neuroglial environment to study cellular mechanisms and evaluate therapeutic targets against these neurodegenerative diseases.

References

[1] B.N. Dugger, D.W. Dickson, Pathology of Neurodegenerative Diseases, Cold Spring Harb. Perspect. Biol. 9 (2017) 1–22. doi:10.1101/cshperspect.a028035.

[2] F.F. Ribeiro, F.J.B. Mendonca, J.B. Ghasemi, H.M. Ishiki, M.T. Scotti, L. Scotti, Docking of Natural Products against Neurodegenerative Diseases: General Concepts, Comb. Chem. High Throughput Screen. 21 (2018) 152–160. doi:10.2174/1386207321666180313130314.

[3] I. Pereira, P. Severino, A.C. Santos, A.M. Silva, E.B. Souto, Colloids and Surfaces B: Biointerfaces Linalool bioactive properties and potential applicability in drug delivery systems, Colloids Surfaces B Biointerfaces. 171 (2018) 566–578. doi:10.1016/j.colsurfb.2018.08.001.

[4] T. Ilc, C. Parage, B. Boachon, N. Navrot, D. Werck-reichhart, Monoterpenol Oxidative Metabolism : Role in Plant Adaptation and Potential Applications, Front. Plant Sci. 7 (2016) 1–16. doi:10.3389/fpls.2016.00509.

[5] R.A. Raguso, More lessons from linalool: Insights gained from a ubiquitous floral volatile, Curr. Opin. Plant Biol. 32 (2016) 31–36. doi:10.1016/j.pbi.2016.05.007.

[6] M. Hoferl, S. Krist, G. Buchbauer, Chirality Influences the Effects of Linalool on Physiological Parameters of Stress, Planta Med. 72 (2006) 1188–1192. doi:10.1055/s-2006-947202.

[7] Y. Sugawara, C. Hara, K. Tamura, T. Fujii, K. Nakamura, T. Masujima, T. Aoki, Sedative effect on humans of inhalation of essential oil of linalool: Sensory evaluation and physiological measurements using optically active linalools, Anal. Chim. Acta. 365 (1998) 293–299. doi:10.1016/S0003-2670(97)00639-9.

[8] D. De Sousa, F. Ferreira, F. Nóbrega, R. de Almeido, Influence of the Chirality of (R)-(-)- and (S)-(+)-carvone in the Central Nervous System: A Comparative Study, Chirality. 19 (2007) 264–268. doi:10.1002/chir.

[9] D.P. De Sousa, F.F.F. Nóbrega, C.C.M.P. Santos, R.N. De Almeida, Anticonvulsant Activity of the Linalool Enantiomers and Racemate: Investigation of Chiral Influence, Nat. Prod. Commun. 5 (2010) 1847–1851.

[10] A.M. Api, D. Belsito, S. Bhatia, M. Bruze, P. Calow, M.L. Dagli, W. Dekant, A.D. Fryer, L. Kromidas, S. La Cava, J.F. Lalko, A. Lapczynski, D.C. Liebler, Y. Miyachi, V.T. Politano, G. Ritacco, D. Salvito, T.W. Schultz, J. Shen, I.G. Sipes, B. Wall, D.K. Wilcox, RIFM fragrance ingredient safety assessment, Linalool, CAS Registry Number 78-70-6, Food Chem. Toxicol. 97 (2015) S29–S38. doi:10.1016/j.fct.2015.12.010.

[11] A.C. Aprotosoaie, M. Hancianu, I. Costache, A. Miron, Linalool : a review on a key odorant molecule with valuable biological properties, Flavour Fragance J. 29 (2014) 193–219. doi:10.1002/ffj.3197.

[12] L.J. Marnett, S.M. Cohen, S. Fukushima, N.J. Gooderham, S.S. Hecht, I.M.C.M. Rietjens, R.L. Smith, T.B. Adams, M. Bastaki, C.L. Harman, M.M. Mcgowen, S. V Taylor, GRASr2 Evaluation of Aliphatic Acyclic and Alicyclic Terpenoid Tertiary Alcohols and Structurally Related Substances Used as Flavoring Ingredients, J. Food Sci. 79 (2014) 428–441. doi:10.1111/1750-3841.12407.

[13] Lewis EM, Oral (gavage) developmental toxicity study of linalool in rats. Charles River Laboratories, Washington, D.C, 2006.

[14] V. Coelho, L. Mazzardo-Martins, D.F. Martins, A.R.S. Santos, L.F. Da Silva Brum, J.N. Picada, P. Pereira, Neurobehavioral and genotoxic evaluation of (-)-linalool in mice, J. Nat. Med. 67 (2013) 876–880. doi:10.1007/s11418-013-0751-6.

[15] S.S. Nascimento, E.A. Camargo, J.M. Desantana, A. Araújo, P. Menezes, W. Lucca, R. Alburquerque, L. Bonjardin, L. Quintans, Linalool and linalool complexed in β-cyclodextrin produce anti-hyperalgesic activity and increase Fos protein expression in animal model for fibromyalgia, Naunyn-Schmiedeberg's Arch Pharmacol. 387 (2014) 935–942. doi:10.1007/s00210-014-1007-z.

[16] S. Mehri, M.A. Meshki, H. Hosseinzadeh, Linalool as a neuroprotective agent against acrylamide-induced neurotoxicity in Wistar rats., Drug Chem. Toxicol. 38 (2015) 162–166. doi:10.3109/01480545.2014.919585.

[17] M. Huo, X. Cui, J. Xue, G. Chi, R. Gao, X. Deng, S. Guan, J. Wei, L.W. Soromou, H. Feng, others, Anti-inflammatory effects of linalool in RAW 264.7 macrophages and lipopolysaccharide-induced lung injury model, J. Surg. Res. 180 (2013) e47--e54.

[18] A. Chadha, K.M. Madyasthat, Metabolism of geraniol and linalool in the rat and effects on liver and lung microsomal enzymes, Xenobiotica. 14 (1984) 365–374. doi:10.3109/00498258409151425.

[19] B. Alinejad, A. Ghorbani, H.R. Sadeghnia, Effects of combinations of curcumin, linalool, rutin, safranal, and thymoquinone on glucose/serum deprivation-induced cell death, Avicenna J. Phytomedicine. 3 (2013) 321.

[20] H. Park, G.H. Seol, S. Ryu, I.Y. Choi, Neuroprotective effects of (-)-linalool against oxygen-glucose deprivation-induced neuronal injury, Arch. Pharm. Res. 39 (2016) 555–564. doi:10.1007/s12272-016-0714-z.

[21] T. Kaur, S. Kaul, A. Bhardwaj, Phytomedicine Efficacy of linalool to ameliorate uremia induced vascular calcification in wistar rats, Phytomedicine. 51 (2018) 191–195. doi:10.1016/j.phymed.2018.10.007.

[22] S. Çelik, A. Ozkaya, Effects of intraperitoneally administered lipoic acid, vitamin E, and linalool on the level of total lipid and fatty acids in guinea pig brain with oxidative stress induced by H 2 O 2, BMB Rep. 35 (2002) 547–552.

[23] M. Ost, C. Doerrier, P. Gama-perez, Analysis of mitochondrial respiratory function in tissue biopsies and blood cells, Curr. Opin. Clin. Nutr. Metab. Care. 21 (2018) 336–342. doi:10.1097/MCO.0000000000000486.

[24] D.H. Jang, S.C. Seeger, M.E. Grady, F.S. Shofer, D.M. Eckmann, Mitochondrial dynamics and respiration within cells with increased open pore cytoskeletal meshes, Co. Biol. 6 (2017) 1831–1839. doi:10.1242/bio.029009.

[25] C. Doerrier, L.F. Garcia-souza, G. Krumschnabel, Y. Wohlfarter, A.F. Mészáros, E. Gnaiger, High-Resolution FluoRespirometry and OXPHOS Protocols for Human Cells, Permeabilized Fibers from Small Biopsies of Muscle, and Isolated Mitochondria, in: Mitochondrial Bioenerg., Springer protocols, 2018: pp. 31–70. doi:10.1007/978-1-4939-7831-1_3.

[26] H. Jun, J.H. Lee, J. Kim, Y. Jia, K.H. Kim, K.Y. Hwang, E.J. Yun, K.R. Do, S. Lee, Linalool is a PPAR ␥ ligand that reduces plasma TG levels and rewires the hepatic transcriptome and plasma metabolome, 55 (2014). doi:10.1194/jlr.M045807.

[27] S.Y. Cho, H.J. Jun, J.H. Lee, Y. Jia, K.H. Kim, S.J. Lee, Linalool reduces the expression of 3-hydroxy-3-methylglutaryl CoA reductase via sterol regulatory element binding protein-2- and ubiquitin-dependent mechanisms, FEBS Lett. 585 (2011) 3289–3296. doi:10.1016/j.febslet.2011.09.012.

[28] B. Rodenak Kladniew, M. Polo, S. Montero Villegas, M. Galle, R. Crespo, M. Garc??a De Bravo, Synergistic antiproliferative and anticholesterogenic effects of linalool, 1,8-cineole, and simvastatin on human cell lines, Chem. Biol. Interact. 214 (2014) 57–68. doi:10.1016/j.cbi.2014.02.013.

[29] B. Cheng, L. Sheen, S. Chang, Hypolipidemic effects of S-(+)-linalool and essential oil from Cinnamomum osmophloeum ct . linalool leaves in mice, J. Tradit. Chinese Med. Sci. 8 (2018) 46–52. doi:10.1016/j.jtcme.2017.02.002.

[30] E. de Almeida, K. Rayane, D.O. Rafael, G. Bosco, L. Couto, A. Beatriz, M. Ishigami, Anxiolytic and Anticonvulsant Effects on Mice of Flavonoids , Linalool , and α -Tocopherol Presents in the Extract of Leaves of Cissus sicyoides L . (Vitaceae), J. Biomed. Biotechnol. (2009) 1–7. doi:10.1155/2009/274740.

[31] V.M. Linck, A.L. da Silva, M. Figueiró, E.B. Caramão, P.R.H. Moreno, E. Elisabetsky, Effects of inhaled Linalool in anxiety, social interaction and aggressive behavior in mice, Phytomedicine. 17 (2010) 679–683. doi:10.1016/j.phymed.2009.10.002.

[32] B.H. Cheng, L.Y. Sheen, S.T. Chang, Evaluation of anxiolytic potency of essential oil and S-(+)-linalool from Cinnamomum osmophloeum ct. linalool leaves in mice, J. Tradit. Complement. Med. 5 (2015) 27–34. doi:10.1016/j.jtcme.2014.10.007.

[33] M. Cline, J.E. Taylor, J. Flores, S. Bracken, S. McCall, T.E. Ceremuga, Investigation of the anxiolytic effects of linalool, a lavender extract, in the male Sprague-Dawley rat., AANA J. 76 (2008) 47–52.

[34] P. Xu, K. Wang, C. Lu, L. Dong, L. Gao, M. Yan, S. Aibai, Y. Yang, X. Liu, Protective effects of linalool against amyloid beta-induced cognitive de fi cits and damages in mice, Life Sci. 174 (2017) 21–27. doi:10.1016/j.lfs.2017.02.010.

[35] M. Chang, Y. Shen, Linalool Exhibits Cytotoxic Effects by Activating Antitumor Immunity, Molecules. 19 (2014) 6694–6706. doi:10.3390/molecules19056694.

[36] A.T. Peana, S. Marzocco, A. Popolo, A. Pinto, (-)-Linalool inhibits in vitro NO formation: Probable involvement in the antinociceptive activity of this monoterpene compound, Life Sci. 78 (2006) 719–723. doi:10.1016/j.lfs.2005.05.065.

[37] Y. Li, O. Lv, F. Zhou, Q. Li, Z. Wu, Y. Zheng, Linalool Inhibits LPS-Induced Inflammation in BV2 Microglia Cells by Activating Nrf2, Neurochem. Res. 40 (2015) 1520–1525. doi:10.1007/s11064-015-1629-7.

[38] K.E. Hopperton, D. Mohammad, M.O. Trépanier, V. Giuliano, R.P. Bazinet, Markers of microglia in post-mortem brain samples from patients with Alzheimer's disease: A systematic review, Mol. Psychiatry. 23 (2018) 177–198. doi:10.1038/mp.2017.246.

[39] S. Jander, M. Schroeter, A. Saleh, Imaging inflammation in acute brain ischemia, Stroke. 38 (2007) 642–645. doi:10.1161/01.STR.0000250048.42916.ad.

[40] R.M. Ritzel, Y.J. Lai, J.D. Crapser, A.R. Patel, A. Schrecengost, J.M. Grenier, N.S. Mancini, A. Patrizz, E.R. Jellison, D. Morales-Scheihing, V.R. Venna, J.K. Kofler, F. Liu, R. Verma, L.D. McCullough, Aging alters the immunological response to ischemic stroke, Acta Neuropathol. 136 (2018) 89–110. doi:10.1007/s00401-018-1859-2.

[41] M. Prinz, S. Jung, J. Priller, Microglia Biology: One Century of Evolving Concepts., Cell. 179 (2019) 292–311. doi:10.1016/j.cell.2019.08.053.

[42] D. Guneykaya, A. Ivanov, D.P. Hernandez, V. Haage, B. Wojtas, N. Meyer, M. Maricos, P. Jordan, A. Buonfiglioli, B. Gielniewski, N. Ochocka, C. Cömert, C. Friedrich, L.S. Artiles, B. Kaminska, P. Mertins, D. Beule, H. Kettenmann, S.A. Wolf, Transcriptional and Translational Differences of Microglia from Male and Female Brains, Cell Rep. 24 (2018) 2773-2783.e6. doi:10.1016/j.celrep.2018.08.001.

[43] M.L. Dubbelaar, L. Kracht, B.J.L. Eggen, E.W.G.M. Boddeke, The Kaleidoscope of Microglial Phenotypes, Front. Immunol. 9 (2018) 1–16. doi:10.3389/fimmu.2018.01753.

[44] P. Douvaras, B. Sun, M. Wang, I. Kruglikov, G. Lallos, M. Zimmer, C. Terrenoire, B. Zhang, S. Gandy, E. Schadt, others, Directed differentiation of human pluripotent stem cells to microglia, Stem Cell Reports. 8 (2017) 1516–1524.

[45] J. Muffat, Y. Li, B. Yuan, M. Mitalipova, A. Omer, S. Corcoran, G. Bakiasi, L.-H. Tsai, P. Aubourg, R.M. Ransohoff, others, Efficient derivation of microglia-like cells from human pluripotent stem cells, Nat. Med. 22 (2016) 1358.

[46] D.A. Lim, A.D. Tramontin, J.M. Trevejo, D.G. Herrera, J.M. García-Verdugo, A. Alvarez-Buylla, Noggin antagonizes BMP signaling to create a niche for adult neurogenesis, Neuron. 28 (2000) 713–726. doi:10.1016/S0896-6273(00)00148-3.

[47] E. Porlan, J.M. Morante-Redolat, M.Á. Marqués-Torrejón, C. Andreu-Agulló, C. Carneiro, E. Gómez-Ibarlucea, A. Soto, A. Vidal, S.R. Ferrón, I. Fariñas, Transcriptional repression of Bmp2 by p21 Waf1/Cip1 links quiescence to neural stem cell maintenance, Nat. Neurosci. 16 (2013) 1567–1575. doi:10.1038/nn.3545.

[48] S.M. Liva, M.A. Kahn, J.M. Dopp, J. De Vellis, Signal transduction pathways induced by GM-CSF in microglia: Significance in the control of proliferation, Glia. 26 (1999) 344–352. doi:10.1002/(SICI)1098-1136(199906)26:4<344::AID-GLIA8>3.0.CO;2-L.

[49] S. Boulakirba, A. Pfeifer, R. Mhaidly, S. Obba, M. Goulard, T. Schmitt, P. Chaintreuil, A. Calleja, N. Furstoss, F. Orange, S. Lacas-Gervais, L. Boyer, S. Marchetti, E. Verhoeyen, F. Luciano, G. Robert, P. Auberger, A. Jacquel, IL-34 and CSF-1 display an equivalent macrophage differentiation ability but a different polarization potential, Sci. Rep. 8 (2018) 1–11. doi:10.1038/s41598-017-18433-4.

[50] E.M. Abud, R.N. Ramirez, E.S. Martinez, L.M. Healy, C.H.H. Nguyen, S.A. Newman, A. V Yeromin, V.M. Scarfone, S.E. Marsh, C. Fimbres, others, iPSC-derived human microglia-like cells to study neurological diseases, Neuron. 94 (2017) 278–293.

[51] B. Popp, M. Krumbiegel, J. Grosch, A. Sommer, S. Uebe, Z. Kohl, S. Plötz, M. Farrell, U. Trautmann, C. Kraus, A.B. Ekici, R. Asadollahi, M. Regensburger, K. Günther, A. Rauch, F. Edenhofer, J. Winkler, B. Winner, A. Reis, Need for high-resolution Genetic Analysis in iPSC: Results and Lessons from the ForIPS Consortium, Sci. Rep. 8 (2018) 1–14. doi:10.1038/s41598-018-35506-0.

[52] P.W. Brownjohn, J. Smith, R. Solanki, E. Lohmann, H. Houlden, J. Hardy, S. Dietmann, F.J. Livesey, Functional Studies of Missense TREM2 Mutations in Human Stem Cell-Derived Microglia, Stem Cell Reports. 10 (2018) 1294–1307. doi:10.1016/j.stemcr.2018.03.003.

[53] A. McQuade, M. Coburn, C.H. Tu, J. Hasselmann, H. Davtyan, M. Blurton-Jones, Development and validation of a simplified method to generate human microglia from pluripotent stem cells, Mol. Neurodegener. 13 (2018) 1–13. doi:10.1186/s13024-018-0297-x.

[54] H. Konttinen, M. e C. Cabral-da-Silva, S. Ohtonen, S. Wojciechowski, A. Shakirzyanova, S. Caligola, R. Giugno, Y. Ishchenko, D. Hernández, M.F. Fazaludeen, S. Eamen, M.G. Budia, I. Fagerlund, F. Scoyni, P. Korhonen, N. Huber, A. Haapasalo, A.W. Hewitt, J. Vickers, G.C. Smith, M. Oksanen, C. Graff, K.M. Kanninen, S. Lehtonen, N. Propson, M.P. Schwartz, A. Pébay, J. Koistinaho, L. Ooi, T. Malm, PSEN1ΔE9, APPswe, and APOE4 Confer Disparate Phenotypes in Human iPSC-Derived Microglia, Stem Cell Reports. 13 (2019) 669–683. doi:10.1016/j.stemcr.2019.08.004.

[55] M.L. Bennett, F.C. Bennett, S.A. Liddelow, B. Ajami, J.L. Zamanian, N.B. Fernhoff, S.B. Mulinyawe, C.J. Bohlen, A. Adil, A. Tucker, I.L. Weissman, E.F. Chang, G. Li, G.A. Grant, M.G. Hayden Gephart, B.A. Barres, New tools for studying microglia in the mouse and human CNS., Proc. Natl. Acad. Sci. U. S. A. 113 (2016) E1738–E1746. doi:10.1073/pnas.1525528113.

[56] O. Butovsky, M.P. Jedrychowski, C.S. Moore, R. Cialic, A.J. Lanser, G. Gabriely, T. Koeglsperger, B. Dake, P.M. Wu, C.E. Doykan, Z. Fanek, L. Liu, Z. Chen, J.D. Rothstein, R.M. Ransohoff, S.P. Gygi, J.P. Antel, H.L. Weiner, Identification of a unique TGF-β-dependent molecular and functional signature in microglia, Nat. Neurosci. 17 (2014) 131–143. doi:10.1038/nn.3599.

[57] H. Pandya, M.J. Shen, D.M. Ichikawa, A.B. Sedlock, Y. Choi, K.R. Johnson, G. Kim, M.A. Brown, A.G. Elkahloun, D. Maric, C.L. Sweeney, S. Gossa, H.L. Malech, D.B. Mcgavern, J.K. Park, Differentiation of human and murine induced pluripotent stem cells to microglia-like cells, (2017) 2–11. doi:10.1038/nn.4534.

[58] P. Garcia-Reitboeck, A. Phillips, T.M. Piers, C. Villegas-Llerena, M. Butler, A. Mallach, C. Rodrigues, C.E. Arber, A. Heslegrave, H. Zetterberg, H. Neumann, S. Neame, H. Houlden, J. Hardy, J.M. Pocock, Human Induced Pluripotent Stem Cell-Derived Microglia-Like Cells Harboring TREM2 Missense Mutations Show Specific Deficits in Phagocytosis, Cell Rep. 24 (2018) 2300–2311. doi:10.1016/j.celrep.2018.07.094.

[59] I. Ferrer, Proteomics and lipidomics in the human brain, in: Handb. Clin. Neurol., 1st ed., Elsevier B.V., 2018: pp. 285–302. doi:10.1016/B978-0-444-63639-3.00020-7.

[60] M.W. Wong, N. Braidy, A. Poljak, The application of lipidomics to biomarker research and pathomechanisms in Alzheimer ' s disease, Curr. Opin. Psychiatry. 30 (2017) 136–144. doi:10.1097/YCO.0000000000000303.

[61] K. Wells, A.A. Farooqui, L. Liss, L.A. Horrocks, Neural Membrane Phospholipids in Alzheimer Disease, Neurochem. Res. 20 (1995) 1329–1333. doi:10.1007/BF00992508.

[62] A. Farooqui, Lipid Mediators and Their Metabolism in the Brain, Springer Science & Business Media, 2011.

[63] R. González-domínguez, T. García-barrera, J.L. Gómez-ariza, Metabolomic study of lipids in serum for biomarker discovery in Alzheimer ' s disease using direct infusion mass spectrometry, J. Pharm. Biomed. Anal. 98 (2014) 321–326. doi:10.1016/j.jpba.2014.05.023.

[64] J.N. Van Der Veen, J.P. Kennelly, S. Wan, J.E. Vance, D.E. Vance, R.L. Jacobs, The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease ☆, Biochim. Biophys. Acta. 1859 (2017) 1558–1572. doi:10.1016/j.bbamem.2017.04.006.

[65] S. Koizumi, S. Yamamoto, T. Hayasaka, Y. Konishi, M. Yamaguchi-Okada, N. Goto-Inoue, Y. Sugiura, M. Setou, H. Namba, Imaging mass spectrometry revealed the production of lyso-phosphatidylcholine in the injured ischemic rat brain, Neuroscience. 168 (2010) 219–225. doi:10.1016/j.neuroscience.2010.03.056.

[66] J.K. Blusztajn, B.E. Slack, T.J. Mellott, Neuroprotective Actions of Dietary Choline, (2017). doi:10.3390/nu9080815.

[67] L. Whiley, A. Sen, J. Heaton, P. Proitsi, D. García-Gómez, R. Leung, N. Smith, M. Thambisetty, I. Kloszewska, P. Mecocci, H. Soininen, M. Tsolaki, B. Vellas, S. Lovestone, C. Legido-Quigley, Evidence of altered phosphatidylcholine metabolism in Alzheimer's disease, Neurobiol. Aging. 35 (2014) 271–278. doi:10.1016/j.neurobiolaging.2013.08.001.

[68] K. Klavins, T. Koal, G. Dallmann, J. Marksteiner, G. Kemmler, C. Humpel, The ratio of phosphatidylcholines to lysophosphatidylcholines in plasma differentiates healthy controls from patients

with Alzheimer's disease and mild cognitive impairment, Alzheimer's Dement. Diagnosis, Assess. Dis. Monit. 1 (2015) 295–302. doi:10.1016/j.dadm.2015.05.003.

[69] G.R. V Hammond, T. Balla, Biochimica et Biophysica Acta Polyphosphoinositide binding domains: Key to inositol lipid biology, BBA - Mol. Cell Biol. Lipids. (2015). doi:10.1016/j.bbalip.2015.02.013.

[70] Y.J. Kim., M.L. Guzman., T. Balla, A highly dynamic ER-derived phosphatidylinositol synthesizing organelle suplies Phosphoinositides to cellular membranes, Dev Cell. 21 (2011) 813–824. doi:10.1016/j.devcel.2011.09.005.A.

[71] M.S. Seren, C. Aldinio, R. Zanoni, A. Leon, F. Nicoletti, Stimulation of Inositol Phospholipid Hydrolysis by Excitatory Amino Acids Is Enhanced in Brain Slices from Vulnerable Regions after Transient Global Ischemia, J. Neurochem. 53 (1989) 1700–1705. doi:10.1111/j.1471-4159.1989.tb09233.x.

[72] G. Divolis, P. Mavroeidi, O. Mavrofrydi, P. Papazafiri, Differential effects of calcium on PI3K-Akt and HIF-1α survival pathways, Cell Biol. Toxicol. 32 (2016) 437–449. doi:10.1007/s10565-016-9345-x.

[73] S.E. Horvath, G. Daum, Lipids of mitochondria, Prog. Lipid Res. 52 (2013) 590–614. doi:10.1016/j.plipres.2013.07.002.

[74] B.-H. Jiang, J.Z. Zheng, M. Aoki, P.K. Vogt, Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells, Proc. Natl. Acad. Sci. 97 (2000) 1749–1753. doi:10.1073/pnas.040560897.

[75] M.J. Glade, K. Smith, Phosphatidylserine and the Human Brain, Nutrition. 31 (2014) 781–786. doi:10.1016/j.nut.2014.10.014.

[76] P. Wood, B. Barnette, J. Kaye, J. Quinn, R. Woltjer, Non-targeted lipidomics of CSF and frontal cortex grey and white matter in control , mild cognitive impairment , and Alzheimer ' s disease subjects, Acta Neuropsychiatr. 27 (2015) 270–8. doi:10.1017/neu.2015.18.

[77] Y.Y. Zhang, L.Q. Yang, L.M. Guo, Effect of phosphatidylserine on memory in patients and rats with Alzheimer's disease, Genet. Mol. Res. 14 (2015) 9325–9333. doi:10.4238/2015.August.10.13.

[78] N. Salem, M. Vandal, F. Calon, The benefit of docosahexaenoic acid for the adult brain in aging and dementia, Prostaglandins Leukot. Essent. Fat. Acids. 92 (2015) 15–22. doi:10.1016/j.plefa.2014.10.003.

[79] A. Farooqui, L. Horrocks, Glycerophospholipids in brain. Phospholipases A2 in neurological disorders, Springer, 2007.

[80] J.M. Dean, I.J. Lodhi, Structural and functional roles of ether lipids, Protein Cell. 9 (2018) 196–206. doi:10.1007/s13238-017-0423-5.

General discussion

APPENDIX

Summary

Neurodegenerative diseases maybe produced by hereditary and sporadic causes, which are characterized by a progressive nervous system dysfunction, between them, Alzheimer's disease (AD) and other ageing-related progressive neurodegenerative disorders, as stroke. The main aim of this work was to help to set up therapeutic targets in neurodegeneration and to identify possible phospholipid biomarkers that reflect the progression of the disease. We demonstrated that oral administration of linalool was able to reduce the main markers in AD (extracellular β-amyloidosis, tauopathy, astrogliosis and microgliosis) and in ischemic stroke the animals had an improvement in motor and cognitive test with a reduction of astrogliosis and microgliosis in the hippocampus. The altered profiles of phospholipids composed of mono and polyunsaturated fatty acids (PC 36:1; 42:1 (24:0/18:1)/LPC 22:6)/ LPE 22:6) in the ischemic hippocampus and the upregulation of PI 36:2 and other LCFA (long chain fatty acids) in the serum of ischemic rats were prevented by linalool. However, we did not know its mechanism of action. For that reason, we investigated whether linalool have a neuroprotective effect on glutamate-induced oxidative stress and affect mitochondrial function. Administration of linalool reduced cell death mediated by glutamate and improved mitochondria morphology. Furthermore, we demonstrated a potential neuroprotective effect of linalool by reduction of mitochondrial ROS formation, mitochondrial calcium uptake, lipid peroxidation and recovery of mitochondrial membrane potential. Besides we showed linalool is able to promote an increase in uncoupled respiration on both high resolution respirometry and Seahorse system. Therefore, we suggest that linalool could represent a therapeutic agent with potential for clinical and translational studies in neurodegenerative diseases.

On the other hand, we evaluated the phospholipid alterations in a two-vessel occlusion (2-VO) model after one month from occlusion. Lipidomic analysis was performed via mass spectrometry in the hippocampus and serum a month postischemia. We found decreases in phospholipids (PLs) associated with neurotransmission, such as phosphatidylcholine (PC) and increases in PLs implied in membrane structure and signaling, such as lysophosphatidylethanolamine (LPE) and phosphatidylserine (PS) in the hippocampus. Complementarily, PC and ether-PC decreased, while Lyso-PC (LPC) and phosphatidylinositol (PI), as neurovascular state sensors, increased in the serum. Taken together, these data suggest inverse PC/LPC-PI levels and PC/LPE-PS as possible biomarkers of postischemic cognitive impairment in rats.

In the same way we investigated potential similarities in lipid profile from post-mortem temporal cortex grey matter, subjacent white matter and cerebrospinal fluid (CSF) of patients with CADASIL and Sporadic Alzheimer's disease (SAD) and control subjects. We observed a significant increase in the total levels of PC and a reduction in LPC in the temporal cortex of dementia groups compared with control group. PS and PI tend to decrease in the gray matter of CADASIL group compared with control group. In the white matter, reductions in phosphatylethanolamine (PE) 32:2 were detected in the dementia groups while in CSF the species more discriminative were PC and its ether derivates as ePE and ePC. Our findings showed a deficiency in LPC and PC in temporal cortex in dementias groups suggesting that those two diseases may have a similar pathophysiology. While alteration in specific phospholipids in white matter and CSF indicate a differential pattern in the fatty acid composition between CADASIL and SAD samples.

In both lipidomic approach we detected interesting changes in phospholipids in ischemic stroke, AD and CADASIL in brain samples, CSF and serum that could help to unravel mechanism in these diseases and help to propose different therapeutic targets to treat transversal pathogenesis.

Resumen

Las enfermedades neurodegenerativas pueden ser de origen hereditario y esporádico, caracterizadas por una disfunción progresiva del sistema nervioso, dentro de ellas se incluyen la enfermedad de Alzheimer (EA) y otras enfermedades neurodegenerativas progresivas, como el accidente cerebrovascular (CVD). El objetivo principal de esta investigación fue ayudar a establecer blancos terapéuticos en Neurodegeneración e identificar posibles marcadores fosfolipídicos que pudieran reflejar la progresión de la enfermedad. Para ello demostramos que la administración oral de linalool redujo los principales marcadores histopatológicos en la EA (β-amiloidosis extracelular, tauopatía, astrogliosis y microgliosis) y en un modelo de isquemia redujo marcadores de inflamación en el hipocampo que correlaciono con la mejoría en las pruebas motoras y cognitivas. Además, el linalool recuperó los perfiles alterados de fosfolípidos compuestos de ácidos grasos mono y poliinsaturados (PC 36: 1; 42: 1 (24: 0/18: 1) / LPC 22: 6) / LPE 22: 6) en el hipocampo y la regulación positiva de diferentes especies del fosfatidilinositol en el suero de ratas isquémicas. Para conocer mejor el mecanismo de acción del linalool, evaluamos su efecto neuroprotector sobre el estrés oxidativo inducido por glutamato en la mitocondria. Nosotros observamos que la exposición de la línea celular HT-22 al linalool redujo la muerte celular por el glutamato en conjunto con la reducción de peroxidación lipídica, especies reactivas y calcio mitocondrial; recuperación del potencial de membrana y aumento de la respiración máxima mitocondrial. Por lo tanto, proponemos al linalool como un agente terapéutico con potencial para estudios clínicos y de translación en enfermedades neurodegenerativas.

Por otro lado, evaluamos las alteraciones de fosfolípidos después de un mes de isquemia cerebral en un modelo de oclusión de dos vasos (2-VO). Encontramos una disminución de la fosfatidilcolina (PC) y un aumento de la lisofosfatidiletanolamina (LPE) y la fosfatidilserina (PS) en el hipocampo, todos asociados con neurotransmisión. Complementariamente, PC y ether-PC disminuyeron, mientras que Lyso-PC (LPC) y fosfatidilinositol (PI) incrementaron en el suero y podrían actuar como sensores del estado neurovascular. En conjunto, estos datos sugieren niveles inversos de PC / LPC-PI y PC / LPE-PS como posibles biomarcadores del deterioro cognitivo post-isquémico en ratas.

Del mismo modo, investigamos posibles similitudes en el perfil lipídico de la sustancia gris de la corteza temporal, la sustancia blanca subyacente post-mortem y el líquido cefalorraquídeo (LCR) de pacientes con CADASIL y la enfermedad de Alzheimer esporádica (SAD) y sujetos control. En donde observamos un aumento significativo en los niveles totales de PC y una reducción en LPC en la corteza temporal de los grupos de demencia en comparación con el grupo control. PS y PI tienden a disminuir en la sustancia gris del grupo CADASIL en comparación con el grupo control. En la sustancia blanca, se detectaron reducciones en la fosfaltidiletanolamina (PE) 32: 2 en los grupos de demencia, mientras que en el LCR las especies más discriminatorias fueron PC y sus derivados de éter como ePE y ePC. Nuestros hallazgos mostraron una deficiencia en LPC y PC en la corteza temporal en grupos de demencias, lo que sugiere que estas dos enfermedades pueden tener una fisiopatología similar, mientras que la alteración de fosfolípidos específicos en la sustancia blanca y el LCR indican un patrón diferencial en la composición de ácidos grasos entre las muestras CADASIL y SAD.

En ambos abordajes lipídicos detectamos cambios interesantes en los fosfolípidos después de una isquemia cerebral, EA y CADASI en muestras de cerebros, LCR y suero que en conjunto pueden ayudar a descubrir los mecanismos involucrados en estas enfermedades y ayudar a proponer diferentes blancos terapéuticos con soluciones transversales

Nederlandse Samenvatting

Neurodegeneratieve ziekten zijn erfelijk of sporadisch. Ze worden gekenmerkt door een progressief niet-functionerend zenuwstelsel in de hersengebieden die betrokken zijn bij de regulatie van beweging en geheugenvorming. De meest voorkomende neurodegeneratieve aandoeningen zijn de ziekte van Alzheimer (AD), een hersenaandoening die vooral het geheugen aantast, ischemische beroerte, en de ziekte van Parkinson die de motorische coördinatie beïnvloedt. Het belangrijkste doel van dit werk was om therapeutische targets voor neurodegeneratie op te zetten en om mogelijke fosfolipide biomarkers te identificeren die de progressie van de ziekte weerspiegelen. We hebben aangetoond dat orale toediening van de terpeen linalool de belangrijkste pathologische markers van AD (extracellulaire β-amyloïdose, tauopathie, astrogliosis en microgliosis) en van een ischemische beroerte (astrogliosis, neuronale dood) kon verminderen. De dieren die behandeld waren met deze terpeen waren beter in motorische en cognitieve tests, met een vermindering van astrogliosis en microgliosis in de hippocampus. Ischemie veroorzaakte een ander profiel van fosfolipiden samengesteld uit enkel- en meervoudig onverzadigde vetzuren (PC 36:1; 42:1 (24:0/18:1)/LPC 22:6)/LPE 22:6) in de ischemische hippocampus en een verhoging van PI 36:2 en andere LCT (lange keten vetzuren) in het serum van ischemische ratten. Linalool voorkwam deze veranderingen in het fosfolipidenprofiel.

Het mechanisme was echter grotendeels onbekend. Daarom hebben we onderzocht of en hoe linalool een neuroprotectief effect zou kunnen hebben tegen door glutamaat-geïnduceerde oxidatieve stress en hoe linalool mitochondriale functie zou kunnen beïnvloeden. Toediening van linalool verminderde celdood veroorzaakt door glutamaat en voorkwam veranderingen in de morfologie en functie van mitochondriën. Verder hebben we een potentieel neuroprotectief effect van linalool aangetoond. Dit neuroprotectief effect bestaat uit een vermindering van mitochondriale ROS-vorming, mitochondriale calciumopname, lipidenperoxidatie en herstel van het mitochondriale membraanpotentiaal. Bovendien hebben we aangetoond dat linalool in staat is om een toename van mitochondriale respiratie te bevorderen, zoals aangetoond door zowel hoge resolutie respirometrie als Seahorse extracellulaire flux analyse. Daarom suggereren we dat linalool een therapeutisch middel zou kunnen zijn met mogelijke implicaties voor klinische en translationele studies tegen neurodegeneratieve ziekten.

Daarnaast evalueerden we de veranderingen in fosfolipiden in een twee-vaten occlusie (2-VO) model, één maand na het begin van de occlusieschade. Van de hippocampus en van het serum dat een maand na de ischemie verzameld was, werd een lipidomische analyse uitgevoerd via massaspectrometrie. In de hippocampus troffen we afnames in fosfolipiden (PL's) aan, die geassocieerd zijn met neurotransmissie, zoals fosfatidylcholine (PC), en toenames in PL's die betrokken zijn bij membraanstructuur en signalering, zoals lysofosfatidylethanolamine (LPE) en fosfatidylserine (PS). Tegelijkertijd namen PC en ether-PC af, terwijl Lyso-PC (LPC) en fosfatidylinositol (PI,) als neurovasculaire sensoren, in het serum toenamen. Tezamen suggereren deze gegevens dat PC/LPC-PI en PC/LPE-PS niveaus als mogelijke biomarkers kunnen dienen voor post-ischemische cognitieve achteruitgang bij ratten.

Om onze bevindingen verder te onderbouwen en onze resultaten van rattenstudies naar menselijke studies te vertalen, hebben we mogelijke overeenkomsten in het lipidenprofiel van post-mortem temporale cortex grijze stof, de onderliggende witte stof en cerebrospinale vloeistof (CSF) onderzocht van patiënten met CADASIL, sporadische Alzheimer (SAD) en leeftijd-gematchte gezonde controle proefpersonen. We zagen een significante toename in de totale PC-waarden en een afname in LPC-waarden in de temporale cortex van dementiegroepen vergeleken met de controlegroep. PS en PI neigen afte nemen in de grijze stof van CADASIL patiënten vergeleken met de controlegroep. In de witte stof werden afnames van fosfatylethanolamine (PE) 32:2 gevonden bij de dementiegroepen, terwijl in CSF de meer discriminerende soort PC was en zijn etherderivaten, ePE en ePC. Onze bevindingen toonden een tekort aan in LPC en PC in de temporale cortex bij dementiegroepen, wat suggereert dat deze twee ziekten mogelijk een vergelijkbare pathofysiologie hebben. Daarnaast duidt de verandering in specifieke fosfolipiden in witte stof en CSF op een verschillend patroon in de vetzuursamenstelling tussen CADASIL- en SAD-monsters.

In beide lipidomische benaderingen hebben we interessante veranderingen gedetecteerd in fosfolipiden in ischemische beroerte-, AD- en CADASIL- brein weefsel, CSF en serum. Deze veranderingen kunnen helpen mechanismen te ontrafelen die bijdragen aan deze ziektes en helpen bij het voorstellen van therapeutische targets om transversale pathogenese te behandelen.

Ten slotte hebben we in een review de rol van microglia bij neurodegeneratieve ziekten laten zien met een update over het huidige model om microglia te bestuderen, met de nadruk op iPSC-technologie. Overeenkomstig presenteren we de resultaten van een differentiatieprotocol van humane microglia, afgeleid van iPSC, en tonen we de volledig ontwikkelde en functionerende capaciteit van deze cellen aan. Kortom, we presenteren verschillende strategieën om neurodegeneratie te modelleren en te bestuderen, en we geven antwoorden op het gebied van inflammatie en lipidomische. Tegelijkertijd geven onze resultaten waardevolle informatie over een nieuw molecuul voor de behandeling van AD en cerebrale ischemie.

General abbreviations

Ach: Acetylcholine
AchE: Acetylcholinesterase
AD: Alzheimer's disease
ApoE: Apolipoprotein E
AA: Arachidonic acid
βA: beta-amyloid
CADASIL: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CBF: Cerebral blood flow
CNS: Central nervous system
COX-2: Cyclooxygenase 2
CSF: Cerebrospinal fluid
CVD: Cerebrovascular diseases
ePC: ether phosphatidylcholine
ePE: ether phosphatidylethanolamine
ePS: ether phosphatidylserine
GSD: Glucose/serum deprivation
GM: Gray matter
Hsp: Heat shock protein
LPC: Lyso-phosphatydilcholine
LPE: Lysophosphatidylethanolamine
LPS: Lysophosphatidylserine
MAP: Microtubule-associated proteins
NFTs: Neurofibrillary tangles
NMDA: N-methyl-D-aspartate
Nrf-2: Nuclear factor erythroid 2-related factor 2
OHSC: Organotypic hippocampal slides cultures
PA: Phosphatidic acid
PC: Phosphatydilcholine
PE: Phosphatydilethanolamine
PE-Cer: Phosphatidylethanolamine Ceramide
PG: Phosphatidylglycerol
PI: Phosphatidylinositol
PLA_2: Phospholipases A_2
PLs: Phospholipids
PLS-DA: Partial least squares-discriminant analysis
PS: Phosphatidylserine
ROS: Reactive Oxygen species
SAD: Sporadic Alzheimer's disease
SM: Sphingomyelin
sMC: Significance multivariate correlation
VIP: Variable importance in projection
WM: White matter

Curriculum vitae

Angelica Maria Sabogal Guáqueta, the author of this thesis, was born in Ibagué, Colombia, on the 12 October in 1987. In 2005, she started her career in Biology at University of Tolima in Colombia. During this period, she developed an interest in Neuroscience and she started to participate in the nursery "Neuroplasticity" in the group Experimental Models for Science Zoohumans directed by Liliana Francis Turner where develop the project "Characterization of the estrous cycle in a population of *Proechimys chrysaeolus* held captive from the group in Experimental Models for Science Zoohumans". During her last year in Biology, she started to participate in the Group of neurodegenerative diseases directed by Angel Cespedes Rubio where developed part of her bachelor thesis called "Effect of the Atorvastatin in different neural populations of neural nigrostriatal system in a model of focal cerebral ischemia with reperfusion" with the co-supervision of Gloria Patricia Cardona Gomez in in the Neuroscience group of Antioquia in University of Antioquia, where she developed her bachelor internship. She obtained her Bachelor degree in December (Summa cum laude) and the Poster Award in the 8th IBRO world congress of Neuroscience and the 1st place prize Enrique Nunez Olarte to research in the area of Basic and Applied Pharmacology.

In 2012, Angélica entered the Biomedical Basic Sciences master at the University of Antioquia, Colombia, where she developed the project called "Neuroprotector effect of quercetin and biflavonoid fraction of *Garcinia madruno* in a triple transgenic model of Alzheimer disease". Her master studies finished in 2014 where Angelica obtained a meritorious thesis. During 2014 she worked with the "Grupo de Investigación en Sustancias Bioactivas" of the University of Antioquia" in the project ""Moléculas lideres a partir de quimiodiversidad natural: estudio integrado de los alcaloides de Amaryllidacea como potenciales neuroprotectores" with the supervision of Edison Osorio.

Following the master degree, the author initiated her PhD studies at the University of Antioquia in January 2015 on the project ""Determination of lipid biomarkers involved in emotional disorder and vascular dementia post-stroke and study of prevention by therapy with natural product" funded by Colciencias with the supervision of Professor Patricia Cardona. In September 2017, she joined the Department of Molecular Pharmacology at the University of Groningen to finish her studies on mechanism of protection in mitochondria by linalool and microglia studies in neurodegeneration under the supervision of Amalia Dolga and Erik Boddeke. The results of which are described in the thesis presented here there are part of the double degree program between University of Antioquia and University of Groningen. During her PhD, Angelica obtained 2 IBRO-grants and 1 Travel grant for Society for Neuroscience.

List of publications

Sabogal-Guáqueta Angélica María, Mayorga-Beltrán Erika Lucia, Gallego García Germán Andreo, Bonilla-Porras Angélica Rocío, Bonilla Ramírez Leonardo, Navarro-Carbonell Doris Elena, De Los Reyes Lina María, Francis-Turner Liliana. Caracterización del ciclo estral y comportamientos asociados en una población de *Proechimys chrysaeolus* mantenidas en cautiverio. Tumbaga. 2013; 8-V.2 13-28.

Sabogal AM, Arango CA, Cardona GP, Céspedes AE. Atorvastatin protects GABAergic and dopaminergic neurons in the nigrostriatal system in an experimental rat model of transient focal cerebral ischemia. Biomédica. 2014;34 (2).

Sabogal-Guáqueta Angélica María, Muñoz-Manco Juan Ignacio, Ramírez-Pineda Jose R, Lamprea-Rodríguez Marisol, Osorio Edison, Cardona-Gómez Gloria Patricia. The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice. Neuropharmacology. 2015;93:134-145.

Sabogal-Guáqueta Angélica María, Osorio Edison, Cardona-Gómez Gloria Patricia. Linalool reverses neuropathological and behavioral impairments in old triple transgenic Alzheimer's mice. Neuropharmacology. 2016;102:111-120.

Sabogal-Guáqueta Angélica María, Osorio Edison, Cardona-Gómez Gloria Patricia. 2017. Chapter 2. Flavonoids in Transgenic Alzheimer's Disease. Book: Neuroprotective Effects of Phytochemicals in Neurological Disorders. 43-63

Sabogal-Guáqueta AM, Carrillo-Hormaza L, Osorio E, Cardona-Gómez GP. Effects of biflavonoids from Garcinia madruno on a triple transgenic mouse model of Alzheimer's disease. Pharmacol Res. 2018.129:128-138

Sabogal-Guaqueta Angélica María, Posada-Duque Rafael, Cortes Natalie Charlotte, Arias-Londoño, Julian D. Cardona-Gómez Gloria Patricia. Changes in the hippocampal and peripheral phospholipid profiles are associated with neurodegeneration hallmarks in a long-term global cerebral ischemia model: Attenuation by Linalool. Neuropharmacology. 2018;135:555-571

Vargas-Restrepo Felipe, Sabogal-Guaqueta Angélica María, Cardona-Gómez Gloria Patricia. Quercetin ameliorates inflammation in CA1 hippocampal region in aged triple transgenic Alzheimer´s disease mice model. Biomedica. 2018; 38:62-9.

Sabogal-Guáqueta Angelica Maria, Villamil-Ortiz Javier Gustavo, Arias-Londoño Julian David and Cardona-Gómez Gloria Patricia. Inverse Phosphatidylcholine/ Phosphatidylinositol Levels as Peripheral Biomarkers and Phosphatidylcholine/ Lysophosphatidylethanolamine-Phosphatidylserine as Hippocampal Indicator of Postischemic Cognitive Impairment in Rats. Frontiers in Neuroscience. 2018; 12:989:1-16

Cortes Natalie Charlotte, Sabogal-Guáqueta Angelica Maria, Cardona-Gómez Gloria Patricia, Edison Osorio. Neuroprotection and improvement of the histopathological and behavioral impairments in a murine Alzheimer's model treated with *Zephyranthes carinata* alkaloids. Biomedicine & Pharmacotherapy. 2019; 110:482–492

Pérez-Corredor Paula, Sabogal-Guáqueta Angelica Maria, Carrillo-Hormaza Luis, Cardona-Gómez Gloria Patricia. Preventive Effect of Quercetin in a Triple Transgenic Alzheimer's Disease Mice Model. Molecules. 2019; 24:1-10

Sabogal-Guáqueta Angélica María, Hobbie Fabian, Keerthi Akshaya, Oun Asmaa, Kortholt Arjan, Boddeke Erik, Dolga Amalia. Linalool attenuates oxidative stress and mitochondrial dysfunction mediated by glutamate and NMDA toxicity. Biomedicine & Pharmacotherapy. 2019; 118:1-12

Acknowledgements

The famous pianist Tom Lehrer said, 'Life is like a piano. What you get out depends on how you play it", that reminds me of my childhood. I was taking piano lessons, and I was thinking to be a musician, but in the end, I played my life in a way where I removed the keyboard. I decided to go down the path of discovery and learn about life, studying Biology. I want to thank my biology teachers at school and high school; in my opinion, you were the most enthusiastic and better ones. To Prof. dr. **Liliana Francis,** who was my first real inspiration to be a scientist ever since the first semester of my career. She later allowed me to join her nursery and her research group. It was a pleasant experience that increased my passion for studying Neuroscience; you introduced me to Prof. dr. **Angel Cespedes** and Prof. dr. **Patricia Cardona,** who were great directors of my bachelor thesis, and thanks to that, we have meaningful results from this research.

I continued playing my notes with professor **Patricia**, my supervisor, for almost 10 years. Thank you for giving me the opportunity to learn and grow inside the Neuroscience group at the University of Antioquia. Thank you for your time, work, dedication, trust, patience, and encouragement, pushing me to take on new challenges and goals. I think that we have nice results and memories over these years. Like the time you were with me at my first Sfn meeting in San Diego, when I was scared with thousands of posters around me, but you calmed down, supported me, and gave me the self-confidence to do a successful presentation of my poster. We had the opportunity to join the different neuroscience meetings in different cities of Colombia; Bogota, Cartagena, and Pereira. All these meetings provided a great opportunity to have discussions and open collaborations and allow the integration integrated the members of the group too. Definitely, you are a source of inspiration and a model as a female researcher in Colombia, and I am entirely grateful for all the support and advice during these years.

Dear dr. **Amalia**, it has been a pleasure to share the last 2 years with you. I would like to express my gratitude towards you as my supervisor. When I decided to do my dual diploma at the University of Groningen, and I had a skype meeting with you, and I discovered the kind and sweet person you are. You are an excellent researcher and you had many projects and ideas in mind for me. During these years, we had a lot of meetings, discussing results, papers, projects and more importantly, you always encouraged to me to be more proactive with new ideas to solve the problems that I was having in the lab. At the same time, you congratulated me when we had good results. For example, when we observed a high percentage of double-positive cells in cell sorting, I could feel your happiness, and for sure, you felt mine ☺. We had time to share nice conversations about life, our countries, and food while sharing a good piece of chocolate; thanks for every gift from your trips and your care about my personal life, offering me a hug when I needed one. You have all my admiration as a person and researcher, and for sure, I will continue learning and sharing with you for future years.

I would like to thank prof. dr. **Erik Boddeke**, first promotor, for accepting me as your student and to allow me to be part of your group. You helped me with administrative things during my PhD. I know that you are super busy, but I am happy that you have some time for regular meetings. Listening to your feedback on my results assisted me a lot in the development of my research.
Special thanks to the members of my tutorial committee, Prof. dr. **Gonzalo Arboleda**, Prof. dr.

Marisol Lamprea, and dr. **Julian Londoño**. Thank you for your valuable insights, suggestions, feedback, and comments during my PhD. Your advice has served me very well and directed my scientific and academic training. Also, to the thesis reading committee, Prof. dr. **Uli Eisel**, Prof. dr. **Carsten Culmsee** and Prof. dr. **Martina Schmidt**, thank you for your comments and valuable suggestions that further improved the quality of this thesis. Dear Martina, particular thanks to you for welcoming me to your department. Thank you for your contagious smile and the birthday cards. I would also like to thank you for encouraging the group to bond during lunch, dinners, and other different activities, such as Sinterklaas. I feel very lucky to be in a department with a strong, female leader, it makes me think that women like you can reach equality in Science.

To my colleagues at the University of Antioquia during my doctorate: **Alejo, Andrea, Gloria, Fredy, Johanna, Hector, Rafael,** thank you for sharing not only the laboratory space but also travels, meals, sadness, and happiness. To the technicians at the University of Antioquia **Claudia** and **Tania**, for your help with ordering and animals in the vivarium. It was essential for the development of my experiments in Colombia. To Hugo and Santi for tasty lunches, aromatic tea, and discussions about life, which helped me to relax after a long and complicated day.

In the department of Biomedical Sciences of Cells & Systems in the UMCG. To all, thank you very much for the support of my experiments there. Special thanks to Prof. dr. **Bart Eggen** for the critical thinking and valuable suggestions for microglia projects; to **Arun** for teaching me the handling of iPSC and valuable recommendations; to **Hilmar** for teaching me the organotypic hippocampal slices cultures technique; to **Nieske** for helping me with the standardization of the protocols with organoids and **Emma** for the transcriptomic analysis in our pilot study; to **Letje** for helping me to cut some organoids in the cryostat. To all the PhD students there: **Yang, Claudio, Leroy, Takuya, Marissa, Chairi, Malte, Laura**, I didn't share too much with you but you seem really nice and passionate about science, so I wish you a lot of good fortune in your future career. I want to thank **Albert Gerding** and Prof. dr. **Barbara Bakker** from UMCG for their support on high-respirometry experiments. Besides, thanks to dr. **Lara Barazzuol** and her student **Danielle** for their collaborations with organoids experiments.

To the Research Group on Bioactive Substances, especially Prof. dr. **Edison Osorio** and his students **Natalie Cortes** and **Luis Carlos Carrillo**, for providing some compounds, for their advice and commitment with our research. Nati, thank you for being my partner in the culture lab in the early mornings, with your expertise and funny jokes too. More than a colleague, you are a good friend, I give you all my love and my best wishes for your scientific career, I will be close to you to celebrate all your successes.

Thanks to Prof. dr. **Reinoud Gosens,** Prof. dr. **Barbro Melgert**, dr. **Loes Kistermarker** and dr. **Hoeke Boersma** for your rigorous attitude and valuable suggestions during my presentations and friendly conversations during lunch, drinks, and pub quizzes.

Thanks to all the people within the Molecular Pharmacology group, for giving me a space to do my research and for bringing me your advice and friendship in an international environment.

My dear **Pien**, words are not enough to thank you for all your kindness and hospitality. You are an exceptional person, and I realized as soon as I met you that you will be my friend and my paranymph. You have been with me during my entire PhD process in Groningen and you are willing to help me with a smile to solve any questions from the laboratory or with any document or procedure in Dutch. Thanks for helping me with iPSC cells and microscopy, and I hope we can participate in more projects together. You know, you are the "famous Pien" for my family and friends because I have so many stories with you. We shared concerts, teather, gardening, picnic, sports, dinners, drinks, but most importantly, hugs at the right moment. You already have a big space in my heart, and you can always count on me.

Dear **Asmaa**, one of the most interesting experiences of living in another country is to be able to establish a friendship with a person who, in the beginning, seemed so different to me. Over time I understood how similar we are as women, though we come from different parts of the world. I value our friendship, having you close to me, in the same office, sharing our daily life. You have been an example of a woman for me, with your full-time work as a mother and doctoral student. Yet, you are always willing to help and offer your smile, even when you are doing multiple things (a superpower). Thanks for your collaboration in seahorse experiments and for helping me when I needed it in the lab, and thank you for agreeing to be my paranymph and share this special moment with me too, and for the hugs, my dear office mate-friend.

Dear **Isabella**, it was a pleasure to share with you the office and other spaces in our daily life in the year you were here. I was quite surprised by the good vibe and complicity that I had with you, sometimes just with our eyes, we understood what we want to say. Our portuñol was perfect when we did not find the perfect word in English to express our thoughts. Obrigada 100% with you, I hope that we can meet soon and just talk as we did in the past. I wish you a lot of good fortune and a fruitful ending for your PhD and in your future career and personal life.

I felt lucky to share with other Brazilians in the lab: **Salomon**, you were so kind as my officemate for my first 2 weeks. It was a pleasure to have lunch with nice discussions about the weather and food. I remember the first time that we saw the snow we were with the MF group in The Crown pub, and we just went outside to enjoy for a short time with the snow. Thanks for enjoying with me beautiful places from Netherlands as Keukenhof and Den Haag. You are a great researcher and friend, I wish you the best in your career future; **Marina**, I just met you since May but since the first weeks, I discovered you are so sweet, generous, intelligent and kind. Thank you for your help and expertise with flow cytometry, small favors in daily life, and to be so collaborative. Besides, to introduce me your family and give the opportunity to share with your babies dogs, I really want that we stayed in contact and collaborate together for a longer time; **Marina** and **Guillerme**, I share little time with you but thank you very much for being so friendly and gentle with me. I wish you all success in your future projects. I can tell all of you that for sure now I feel the brother country Brazil closer to me thanks to you.

Inge, thank you very much for welcome and introduction to the lab in the summer of 2017; we had intense work for 2 weeks doing Oroboros experiments, but in the wait time, we got distracted getting to know each other, and I saw you are a sweet and intelligent person. Thank

to help to find the reagents, protocols, and equipment in the lab, but for share dinners, like the Colombian "patacones", conferences and even concerts like watching "The killers" in the coldest day in Amsterdam ☺. I wish you the best in your future defense and personal and professional life.

Ahya and **Chiara**, thanks for your kindness and offer me your friendship, to have the opportunity to share picnics, dinners with interesting conversations where again I discover how being from different continents, we share so many preferences like series, books, and political opinions. I hope to share much more in the near future. Thanks for all the helps you provided in my PhD.

Mariska, thanks to being in charge of so many things in the lab, I really appreciate that you put in my place a welcome paper without even knowing me, you are always aware on birthdays, changes in the lab, schedules and bringing delicious homemade cakes thanks for all these details that are so important to maintain the group harmony. I know that you are having a wonderful moment now with your little Bernd, I wish you all happiness and health for your lovely family.

Xinhui, "selfie girl," thanks to capturing a lot of god moments for the group, to do activities searching for integration. You are a kind and enthusiastic person with a lot of funny stories, to offer your house for my first "hot pot" it was really delicious, a little spicy but I just have good memories for that night and other dinners, picnics and drinks that we share. I wish you all the best for your future.

To the new PhD students: **Alejandro**, **Dan, Shanshan, Habibi, Rosa, Anienke, Laura, Gwenda,** and **Tiago,** I did not share so much with you yet, but you seem nice and enthusiastic persons. I wish you a lot of success in your projects and personal life.

Birgit, thanks to introducing the HT-22 cell´s world, your patience and help during my first weeks in the lab help me to use the dynamics. It was nice to see you having your PhD defense successfully, and thanks again to invited me to your house in Bonn during the Carnival, I really enjoy it. I know that you are doing a great job and I am sure that you will continue it. **Haoxiao,** you change my opinion about shy Chinese, you have so many strong opinions in Science and life, you are so nice and considerate. Thanks for your help in normal daily life in the lab. I wish you a lot of happiness with the little Alice and your husband and success in your professional career.

Annet, it was really good to share the office with you. Even with short conversations with you, but I always feel that you transmit inner peace, and calm around you and you honor the sign on our door "stress free zone". I wish you the best for you always: a lot of health and love. **Nshunge** you are a very outgoing person, perfect for chatting due to thousands of stories that you have to share, your strong laugh can be contagious, so thanks for bringing your energy to the office, now you are in another floor but I hope to share some a lot more coffees with you. I wish you a lot of happiness, love, and prosperity to your lovely family.

I would like to say thanks **to Carolina, Manon, Melissa, Sophie,** and **Vasiliki**; even when you are not familiar with my brain studies, you were available for questions and offering their help on protocols, substances and organizing things in the lab at any time.

Janneke, **Trix** and **Diana** from **bcn** thank you for offering help on administrative things, sending packages, schedule appointments, and organizing reimbursements.

I was my pleasure to be a co-supervisor of bachelor and master students: **Angelita, Paula** and **Mariana** at the University of Antioquia and **Fabian, Akshaya** and **Vitor** at the University of Groningen, different nationalities, but all of them enthusiastic and proactive students that help me in different projects during my PhD, thanks to your support and nice moments. I am really happy that I keep the contact with all of you, and to see that you are now starting your PhD, planning to do it or finishing your medicine bachelor with excellent grades makes me feel proud of all you. You are outstanding students and friends; I wish you all the best in your professional and personal life.

Javi, my friend, it has been very interesting to see you grow personally and professionally, I still have the memory of 10 years ago, the long-hair boy full of dreams with an excellent sense of humor and I am glad to see be your dreams come true. Thank you for being my host during my first 2 weeks and guiding me in the adaptation process at the UMCG to always have some minutes to have a coffee and the curious discussions about the realities surround us. It will be a pleasure to continue seeing how you continue achieving everything you set in your mind, I wish a lot of happiness with Carling. **Liz,** it has been short time since you are living in Groningen, but we share a lot in our master in Colombia and is quite impressive observe how similar thoughts and likes we have, I know that you will do a great work during the next years in your PhD, I hope that friendship will strengthen too.

To other Colombians that I have the opportunity to know in Groningen: **Monica, Caro, Anita, Cata, Isabel, Angelica, Ailine, Juan Manuel and Valentina** thanks to share trips, dinners and nice conversations in our beautiful Spanish language. I wish you a lot of success in your life.

Familia y amigos

Sin lugar ha duda, hoy no estaría finalizando este importante paso en mi vida si no hicieran parte de mi vida, ustedes son mi gran motivación para levantarme cada día y desear ser una mejor persona. **Mama** y **papa**: los amo con todo mi corazón, gracias por su compañía, por consentirme tanto, por sus palabras, por instruirme y solo obligarme a una cosa: seguir mis sueños. Mucha gente por estos lados siempre se sorprende cuando les digo que hablo con ustedes casi a diario, pero es que el saber como están y recordarles lo mucho que los amo, nos permite dormir más tranquilamente.

A ti mi querida hermana, mi Lore preciosa, **sis**, no sabes cuanto agradezco a mis padres y a Dios por tu vida en la mía, tu eres mi amiga, confidente, compañera de proyectos, de viajes: la persona con la que sencillamente puedo contar siempre. En medio de dificultades nos hemos levantando la una a la otra y el estar siempre disponible para la otra es un lujo que no todos tienen. Gracias

por motivarme y acompañarme durante este proceso académico desde la distancia. Estuve muy feliz de que tu y mi madre me acompañaran en mi defensa en Medellín y no te imaginas cuanto más de que también puedan estar aqui ☺. Espero estar cerca de ti para seguir viendo como conquistas tus sueños y el mundo a su paso, te amo inmensamente.

A mi tío **Alberto**, **prim@s**, **tí@s**, a mi abuelita **Carmen**, por gracias por estar siempre pendiente de mi y demostrarme su amor cada vez que es posible con un abrazo, con una comida típica tolimense como lo es el tamal o con el arequipe que con tanto amor me hace mi abue, de verdad estoy eternamente agradecida con cada uno de ustedes. Y ahora a mis sobrinas Isabella y Valeria, que en realidad son mis primas segundas, pero que desde la conocí por primera vez me enamoraron como nunca lo imaginé; y que cuando salen corriendo hacia mi a abrazarme gritándome tía Angie me siguen enamorando, las amo chiquitas. Me encanta ver como crecen y como se han convertido en unas niñas inteligentes, hermosas y únicas; siempre estaré pendiente de ustedes y espero estar de una u otra manera siempre que me necesiten. Gracias, Dios por tus innumerables bendiciones a través de mi familia.

A mi grupo de amigos desde el colegio: **Elizabeth**, amiga tu sabes lo mucho que te aprecio y creo que a pesar de los kilómetros me conoces mejor que nadie en el mundo, gracias por siempre estar dispuesta a escucharme, aconsejarme y porque no regañarme ☺, por ser mi mejor amiga; tu sabes que tienes un lugar muy grande en mi corazón, que ahora se ha ampliado con la llegada de Tomas y Matias. **Diana**, ami, gracias por tu sinceridad y pragmatismo en nuestras conversaciones, por el apoyo incondicional que me has brindado siempre, ha sido un placer encontrarme contigo en la universidad y que nos convirtiéramos en biólogas juntas. **Caro, Maria** y **Os**: gracias por compartir tantos momentos especiales, espero nos sigamos reuniendo a recordar nuestras viejas historias, pero también creándolas.

A mis amig@s con los que he mantenido el contacto a pesar de la distancia y me escuchan siempre y están pendiente de mi, deseándome lo mejor: **Laurita, Lu, Adriana, Camilo, Erika, Laurita Canasto** y tanta otra gente con la que tengo el placer de compartir mi vida, gracias por tantas tertulias llenas de sonrisas, anécdotas, discusiones de tinte político o científico, o conversaciones banales que simplemente nos distraían, pero también por sus sabios consejos, en fin por compartir esta etapa maravillosa de mi vida. **Cris**, muchas gracias por tu compañia, las cenas, los viajes, las criticas constructivas a mi trabajo, de verdad que han sido muchos los buenos y significativos momentos contigo. A todos les deseo muchos éxitos y felicidad en su futuro, los quiero y llevo en mi corazón.

I feel very fortunate to be surrounded by a large number of colleagues, friends, and family without whom this thesis as a result of my PhD would not have been possible. To all of you, my sincere acknowledgments to be there and contribute during my PhD.