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# Age-dependent changes in nervonic acid-containing sphingolipids in mouse hippocampus

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ABSTRACT

Sphingolipids have been implicated in age-related neurodegeneration. Previous studies have reported elevated ceramide levels in the brain of old rodents, but a systematic investigation of the impact of age on brain sphingolipid metabolism is still lacking. Here we quantified 17 key sphingolipid species in the hippocampus of young (3 months), middle-aged (12 months) and old (21 months) male and female mice. Lipids were extracted and quantified by liquid chromatography/mass spectrometry; transcription of enzymes involved in sphingolipid biosynthesis was evaluated by qPCR. Age-dependent changes of multiple sphingolipid species - including ceramide (d18:1/18:0), sphingomyelin (d34:1), hexosylceramide (d18:1/16:0), ceramide (d18:1/24:0) - were found in mice of both sexes. Moreover, sex-dependent changes were seen with hexosylceramide (d18:1/18:0), ceramide (d18:1/22:0), sphingomyelin (d36:1) and sphingomyelin (d42:1). Importantly, an age-dependent accumulation of sphingolipids containing nervonic acid (24:1) was observed in 21 month-old male ($p = 0.04$) and female mice ($p < 0.0001$). Consistent with this increase, transcription of the nervonic acid-synthesizing enzyme, stearoyl-CoA desaturase (*Scd1* and *Scd2*), was upregulated in 21 month-old female mice (*Scd1* $p = 0.006$; *Scd2* $p = 0.009$); a similar trend was observed in males (*Scd1* $p = 0.07$). In conclusion, the results suggest that aging is associated with profound sex-dependent and -independent changes in hippocampal sphingolipid profile. The results also highlight the need to examine the contribution of sphingolipids, and particularly of those containing nervonic acid, in normal and pathological brain aging.

## 1. Introduction

Sphingolipids are a class of bioactive signaling molecules that regulate key cellular processes including cell growth, senescence and apoptosis [1–4]. Ceramides occupy a central place in sphingolipid metabolism (Fig. 1): they are synthesized *de novo* from the condensation of serine and palmitoyl-coenzyme A by the action of serine palmitoyltransferase (SPT), from the reacylation of sphingoid long-chain bases, or from the breakdown of more complex sphingolipids such as sphingomyelins and hexosylceramides [5]. Moreover, ceramides give rise to sphingosine, which is phosphorylated by sphingosine kinases to produce the transcellular messenger sphingosine-1-phosphate [6].

Evidence from animal and human studies suggests that Alzheimer's disease (AD) and Parkinson's disease (PD) are associated with abnormalities in sphingolipid metabolism [7–10]. Aging is the primary risk factor for AD and PD [11,12]. Multiple lines of evidence indicate that tissue ceramide profiles change during aging and in response to a variety of age-related stress factors (e.g. oxidative stress) [8,13–16]. Despite this growing body of evidence, a systematic study of the impact of age on brain sphingolipid metabolism remains to be performed. Such a study should take into consideration as an independent variable sex along with age, because of the dimorphic trajectory of both healthy aging and neurodegenerative disorders [17,18].

In the present study, we used a targeted lipidomic approach to

*Abbreviations:* AD, Alzheimer's disease; PD, Parkinson's disease; SPT, serine palmitoyltransferase; TFA, trifluoroacetic acid; LC/MS, liquid chromatography/mass spectrometry; UPLC, ultra-performance liquid chromatography; TQ-MS, triple quadrupole mass spectrometer; ESI, electrospray ionization; MRM, multiple reaction monitoring; Lass1, Lag1 longevity assurance homolog 1; Lass2, Lag1 longevity assurance homolog 2; Scd1, stearoyl-CoA desaturase 1; Scd2, stearoyl-CoA desaturase 2; Gba1, glucosylceramidase; Scarb2, scavenger receptor class B member 2; Psap, prosaposin; Galc, galactosylceramidase; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Hprt, hypoxanthine phosphoribosyltransferase; SEM, standard error of the mean; ANOVA, analysis of variance; CerS, ceramide synthase; LIMP2, lysosome membrane protein 2; CNS, central nervous system; DLB, Dementia with Lewy Body; MUFA, monounsaturated fatty acid; Elovl1, elongase 1; Elovl6, elongase 6

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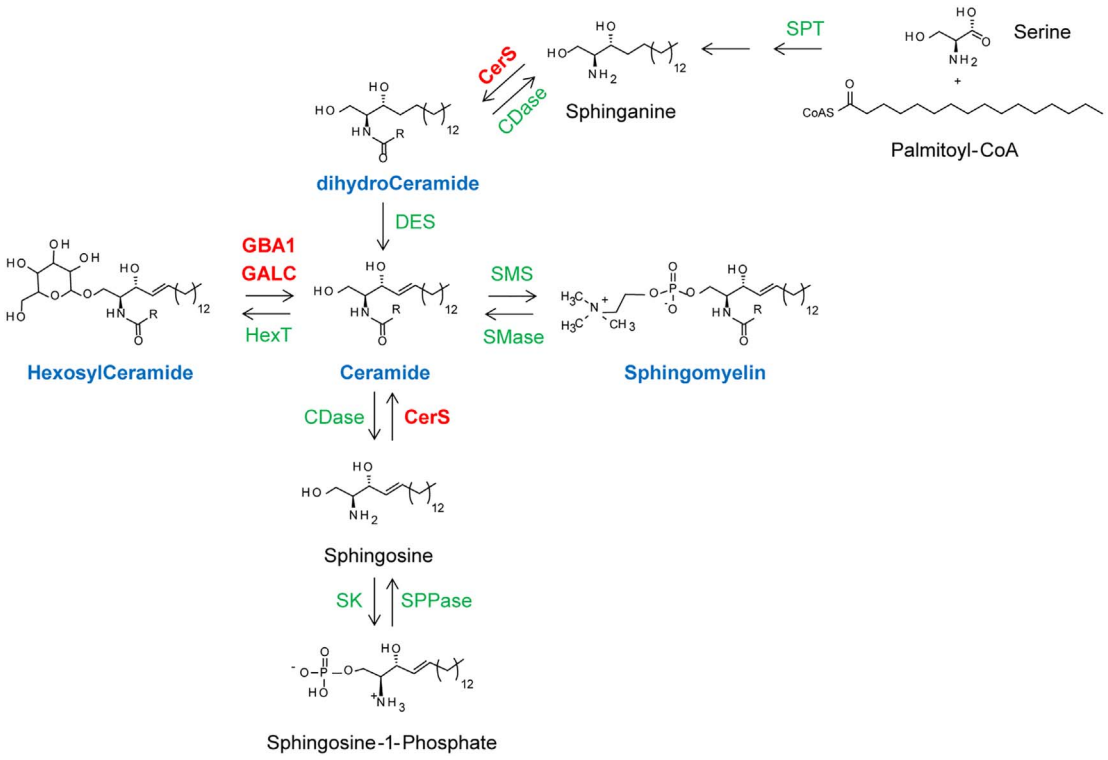


**Fig. 1.** Schematic view of sphingolipid metabolism, highlighting metabolites and enzymes targeted by the present study. Abbreviations: SPT (serine palmitoyltransferase); CerS (ceramide synthase); CDase (ceramidase); DES (desaturase); GBA1 (glucosylceramidase); GALC (galactosylceramidase); HexT (hexosyltransferase); SMS (sphingomyelin synthase); SMase (sphingomyelinase); SK (sphingosine kinase); SPPase (sphingosine-1-phosphate phosphatase).

identify age- and sex-dependent alterations in sphingolipid metabolism in mouse hippocampus. We selected this brain region because it is one of the first to become damaged in AD, leading to memory loss and cognitive impairment [19]. The results suggest that aging is accompanied by multiple, sexually dimorphic changes in hippocampal sphingolipid metabolism. Notably, we found that sphingolipid species containing the long-chain monounsaturated fatty acid, nervonic acid (24:1), are markedly affected by aging, pointing to these lipids as potential contributors to age-dependent cognitive impairment.

## 2. Materials and methods

### 2.1. Animals

Male and female C57Bl/6J mice (3, 12 and 21 months) were purchased from Charles River Laboratories (Calco, Lecco, Italy). Upon arrival, they were acclimatized to the vivarium and kept in a temperature (22 °C) and humidity controlled environment under a 12 h light/12 h dark cycle (lights on at 7:00 A.M.). Animals were housed under enriched conditions [20,21] with bedding changes every 15 days in mice aged 3–12 months, and every 21 days in mice aged 21 months. Standard chow and water were available *ad libitum*. All procedures were performed in accordance with the Ethical Guidelines of the European Community Council (Directive 2010/63/EU of 22 September 2010) and accepted by the Italian Ministry of Health.

### 2.2. Chemicals

Sphingolipid standards were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Solvents were from Sigma-Aldrich (Milan, Italy).

### 2.3. Tissue collection

Mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Brains were removed; hippocampi were dissected on an ice-cold glass plate and were immediately flash frozen in liquid $N_2$. Samples were stored at − 80 °C before analyses.

### 2.4. Lipid extraction

Lipid extractions were performed according to a modified Bligh and Dyer protocol, as previously reported [22]. Briefly, frozen hippocampi (10–20 mg) were homogenized in 2 mL of a methanol/chloroform mixture (2:1 vol/vol) containing trifluoroacetic acid (TFA, 0.1% final concentration), and spiked with a mixture of internal standards consisting of the following unnatural odd-chain lipids: 200 nM ceramide (d18:1/17:0), 400 nM sphingomyelin (d18:1/17:0) and 500 nM heptadecanoic acid (17:0). Glucosylceramide (d18:1/12:0) 200 nM was added as internal standard for hexosylceramides after having verified the absence from hippocampal extracts. After mixing for 30 s, lipids were extracted with chloroform (0.6 mL) and extracts were washed with purified water (0.6 mL). Samples were centrifuged for 15 min at 2800 × *g* at 15 °C. After centrifugation, the organic phases were collected and transferred to a new set of glass vials. To increase the extraction efficiency, the aqueous fractions were subjected to a second extraction. The organic phases were pooled, dried under $N_2$ and residues were dissolved in 0.2 mL of methanol/chloroform (9:1 vol/vol). 0.1 mL of the total extract was saved for the measurement of more polar analytes such as sphingomyelins and hexosylceramides. The remaining solvent was evaporated under $N_2$. Lipids were reconstituted in chloroform (2 mL) and fractionated using small glass columns packed with Silica Gel G (60-Å 230–400 Mesh ASTM; Whatman, Clifton, NJ). Ceramides and fatty acids were eluted with 2 mL of chloroform/methanol (9:1 vol/vol). The solvent was evaporated under $N_2$; dried material was