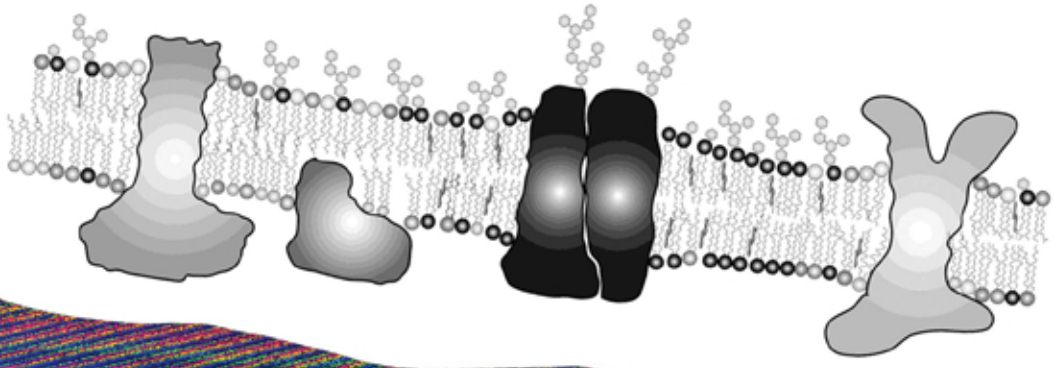
Cecilia L. Watkins
Editor

# Sphingomyelin and Ceramides

## Occurrence, Biosynthesis and Role in Disease



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## OCCURRENCE, BIOSYNTHESIS AND ROLE IN DISEASE

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# SPHINGOMYELIN AND CERAMIDES

## OCCURRENCE, BIOSYNTHESIS AND ROLE IN DISEASE

CECILIA L. WATKINS
EDITOR


*New York*

## NOTICE TO THE READER



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# PREFACE

In this book, exciting new approaches that open a window to further characterize sphingolipid-enriched domains in cell membranes during both physiological and pathological processes are reviewed. Furthermore, sphingolipids (SLs) are especially important in the central nervous system (CNS) where they are a necessary structural component of membranes of brain cells or signaling molecules. Ceramides are the core constituent of most sphingolipids. The authors review the possible sources of ceramides in the central nervous system (CNS) and present information about recent preclinical clinical trials of therapies targeting the ceramide pathway in the brain. Data about significant alteration in levels of ceramides in brain cells during the development of different neurodegenerative disease are also discussed, such as in with Alzheimer's disease, Parkinson's disease multiple sclerosis, cerebral ischemia, Gaucher's and Farber's disease.

Chapter 1 – Sphingolipids are involved in a wide range of physiological and pathological processes none only as signaling molecules but also as key structural components regulating the lateral organization of cellular membranes. The preferential interaction of these biomolecules with cholesterol support the actual theory related with membrane heterogeneity in vivo, the raft theory. Rafts are believed to be highly-dynamic and small domains enriched in sphingolipids, cholesterol and certain proteins present in the membrane of cells. The idea of these domains compartmentalizing cellular processes is a central hypothesis in biomedical research from immunology, virology, neurobiology to cancer.

The use of microscopy to study lateral heterogeneity in biological membranes was developed during the nineties with the use of artificial models systems such as giant unillamelar vesicles and supported-lipid bilayers. The

combination of confocal and two-photon microscopy techniques with fluorescent and solvatochromic probes like Laurdan enabled the acquisition of spatially-resolved information about the fluidity and/or order of artificial bilayers showing phase segregation. The development of new techniques combining Laurdan imaging with fluorescence fluctuation spectroscopy allowed the detection of highly-packed microdomains in natural cell membranes.

In this article the authors review these exciting new approaches that open a window to further characterize these sphingolipid-enriched domains in cell membranes during both physiological and pathological processes.

Chapter 2 – Sphingolipids (SLs) are especially important in the central nervous system (CNS) where they are necessary structural component of membranes of brain cells or signaling molecules. Homeostasis of membrane sphingolipids in neurons and myelin is essential to preventing the loss of synaptic plasticity, cell death and neurodegeneration. Equilibrium of balance between specific SLs is essential for normal neuronal function. Even minor changes in the SLs balance can have dramatic effect on neurological and behavioral deficiencies. Over the past decade, it was found that relatively simple SLs, such as ceramide, sphingosine, sphingosine-1-phosphate and glucosylceramide play important roles in neuronal functions by regulating rates of neuronal growth, differentiation and death. Inducible dysfunction of the ceramide pathway, which is abundant in the brain as well as in peripheral organs, may account for neuronal desorders, behavioral symptoms, and further promote inflammation and oxidative stress.

Ceramides are the core constituent of most sphingolipids. They can be produced by hydrolysis of sphingomyelin (SM) via sphingomyelinases (SMases) or synthesized de novo from fatty acyl CoA and sphingosine. Ceramides are important second messenger molecules that regulate diverse cellular processes including cell growth, differentiation, and apoptosis. Ceramide levels in CNS also increase in response to aging and various age-related stress factors and are directly involved in apoptotic signaling in various neuronal cells, including neurons.

Because ceramides are so important as signalling components in the CNS, changes in brain ceramides levels due to their increased or decreased synthesis or metabolism may result in homeostatic dysregulation and ultimately neurodegeneration. This is extremely important because neurodegeneration is a characteristic component of all dementias.

In their review the authors discuss the possible sources of ceramides in CNS (1); summarize data about significant alteration in levels of ceramides in

brain cells during development of different neurodegenerative disease such as Alzheimer's disease, Parkinson disease, multiple sclerosis, cerebral ischemia, Gaucher's, Farber's diseases, and etc. (2); present information about recent preclinical and clinical trials of therapies targeting ceramide pathway in brain.

Still it is currently unknown if ceramides are associated with CNS diseases through a direct or indirect mechanism. However, it is important to further study and confirm the role of these lipids in CNS (neurodegenerative) diseases as this would suggest possible modifiable risk factors that may serve as targets for strategies of prevention.

Chapter 3 – Alzheimer's disease (AD), the most common leading form of dementia in elderly people, is a chronic and progressive neurodegenerative disorder. The most frequently investigated neuropathological hallmarks of AD are extracellular deposits of neurotoxic amyloid β peptide (Aβ) and intracellular aggregates of hyperphosphorylated tau protein. Ceramides, the major molecules of sphingolipid metabolism, have been linked to AD susceptibility and pathogenesis, including both Aβ pathology and tau aggregation. Elevated levels of ceramides directly increase Aβ levels acting on β-secretase, a key enzyme in the proteolytic cleavage of Aβ precursor protein (APP). In turn, soluble and fibrillar forms of Aβ activate sphingomyelinases, enzymes that catalyze the breakdown of sphingomyelin to ceramides, and lead to further increase in Aβ generation. Ceramides are also linked to tau phosphorylation, in particular by modulating activity of protein phosphatase 2A, the major tau phosphatase in the human brain. Hence, preservation of neuronal ceramide homeostasis is of major importance for normal brain functioning. This chapter summarizes recent findings and potential targets for novel therapeutic approaches in AD regarding described devastating Aβ-ceramides-tau cascade.

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*Chapter 1*

# NEW FLUORESCENCE MICROSCOPY APPROACHES TO EXPLORE THE INFLUENCE OF SPHINGOLIPIDS ON LATERAL ORGANIZATION OF BIOMEMBRANES: FROM ARTIFICIAL SYSTEMS TO CELLULAR MEMBRANES

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## ABSTRACT

Sphingolipids are involved in a wide range of physiological and pathological processes none only as signaling molecules but also as key structural components regulating the lateral organization of cellular membranes. The preferential interaction of these biomolecules with cholesterol support the actual theory related with membrane heterogeneity in vivo, the raft theory. Rafts are believed to be highly-dynamic and small domains enriched in sphingolipids, cholesterol and certain proteins present in the membrane of cells. The idea of these domains compartmentalizing cellular processes is a central hypothesis in biomedical research from immunology, virology, neurobiology to cancer.

The use of microscopy to study lateral heterogeneity in biological membranes was developed during the nineties with the use of artificial models systems such as giant unillamelar vesicles and supported-lipid bilayers. The combination of confocal and two-photon microscopy techniques with fluorescent and solvatochromic probes like Laurdan enabled the acquisition of spatially-resolved information about the fluidity and/or order of artificial bilayers showing phase segregation. The development of new techniques combining Laurdan imaging with fluorescence fluctuation spectroscopy allowed the detection of highly-packed microdomains in natural cell membranes.

In this article we review these exciting new approaches that open a window to further characterize these sphingolipid-enriched domains in cell membranes during both physiological and pathological processes.


## SPHINGOLIPIDS AND LATERAL ORGANIZATION OF MEMBRANES

Biomembranes define cellular compartments and constitute the boundary between the inner space and the external milieu. Far from being just a passive barrier, membranes constitute a highly dynamic structure responsible for regulating essential biochemical processes of the cell. [1].

The description of the structure and dynamics of biomembranes has evolved from the original fluid-mosaic model proposed by Singer and Nicolson [2] toward a more complex description that considers a huge variety of components with complex and dynamic lateral interactions (Figure 1). These interactions originate relatively transient or more stable structures enriched in certain membrane components that play relevant roles in cellular

processes such as signaling in immunological synapses [3, 4], membrane trafficking [5, 6] and viral infection cycles. [7-9]

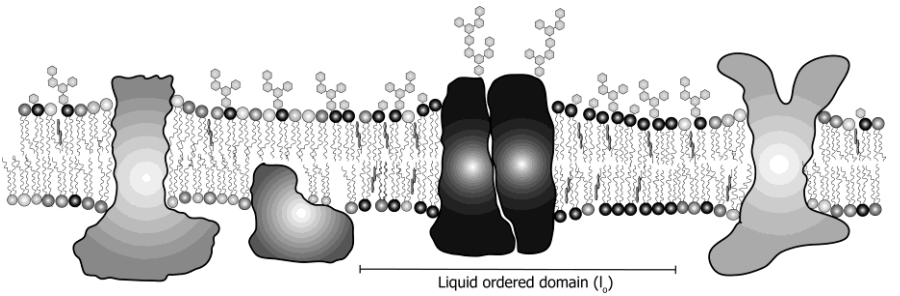


Figure 1. Lateral organization of biological membranes. Cartoon representing the crowding of the plasma membrane and the lateral heterogeneity induced by the different biophysical properties of phospholipids, sphingolipids (depicted in dark gray) and membrane proteins. Rafts are liquid ordered (lo) domains enriched in sphingolipids, cholesterol and certain membrane proteins.

In addition to the role of sphingolipids in physiological and pathological signaling, these lipids are essential structural components of biological membranes playing a key function on their lateral organization. Sphingolipids preferentially interact with cholesterol in biological membranes leading to the formation of clusters named rafts [10] defined as "small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes". [11] Rafts are able to form larger and more stable platforms upon specific signals. [12]

Given the relevance of these membrane structures to cell function, a significant effort have been done to explore the principles governing the lateral segregation of membrane components. In the following sections we briefly describe the systems as well as the spectroscopic and microscopic techniques used in these studies focusing our analysis on those involving sphingolipid-enriched rafts.

## EXPLORING LATERAL ORGANIZATION: FROM ARTIFICIAL TO NATURAL BILAYERS

Different models of biological membranes have been used to explore the physical basis of cell membrane organization and function. The big advantage of these systems is the possibility of changing their composition and

quantitatively characterizing their properties, including the segregation of lipid phases with different physical properties.

In early years researchers used suspensions of liposomes (e.g., small unilamellar vesicles and large unilamellar vesicles, sizing ~30 nm and ~120 nm, respectively [13]), and multilamellar vesicles. [14] The small size of these liposomes did not allow studying the lateral organization of single liposomes rather; their properties were assayed by bulk techniques such as spectrofluorometry [15], isothermal titration and differential scanning calorimetry (ITC and DSC) [16, 17] and electron spin resonance (ESR) [18] that provide the average properties of a large number of lipid vesicles.

To explore the multiscale level of complexity of natural membranes, researchers used methods that could provide dynamic information of biomembranes with high spatial resolution. The combined use of supported lipid bilayers (SLB) or giant unilamelar vesicles (GUVs) with modern fluorescence microscopy tools constituted a new way of exploring in situ the organization of biomembranes.

Supported lipid bilayers (SLB) consist of a lipid bilayer adsorbed onto the surface of a suitable solid substrate. There are different methods to prepare these bilayers (reviewed in [19]); the simplest one consists on spreading small lipid vesicles on a hydrophilic substrate. In this one-step procedure vesicles fuse onto the surface forming a lipid bilayer by self-assembly. A main concern when using SLBs is that the close proximity of the substrate to the membrane may affect the bilayer properties. While Johnson [20] determined that a 10–20 Å water layer containing ions separates the lipid layer and the solid support, recent studies showed that the solid support may interact with the closest leaflet of the membrane and introduce some segregation of membrane components. [21]

Giant unilamellar vesicles (GUVs) constitute an attractive alternative to SLB since they interact with a surface in a very small region and are considered as free standing bilayers (reviewed in [22]). The radii of these vesicles (5-50 μm) are in the order of those observed in eukaryotic cells and thus their curvatures are very similar. Also, they can be easily imaged in microscopes, allowing their study as individual liposomes. [23] There are several methods to prepare GUVs [22]; the most commonly used protocols are based on the electroformation method described by Angelova et al., [24, 25]. Briefly, a solution of the selected lipids in chloroform is spread over two Pt electrodes and dried to remove any traces of the solvent. The electrodes are mounted in an observation chamber and after adding the appropriate buffer, they are connected to a function generator that applies a sine function

(amplitude, 2-3V; frequency, 10 Hz) to generate the GUVs. It is extremely important to set the temperature of the system to 10°C or more over the corresponding transition temperature of the lipids being used. [26] Modifications of this protocol have been introduced to allow the generation of GUVs from natural membranes in more physiological conditions (see for example, [27]).

As we will further discuss below, these model systems allowed for the first time the visualization of large domain segregation in raft-like mixtures of lipids. [28]

## FLUORESCENCE AS A TOOL TO REVEAL LIPID ORGANIZATION IN MEMBRANES

The number of fluorescent probes designed for the detection of membrane rafts is growing rapidly. These probes can be arbitrarily classified into 3 different groups: i) those that specifically label lipid components in the membrane, ii) probes that selectively partition to certain lipid domains and finally, iii) fluorescent probes that sense certain properties of their microenvironment and thus provide additional information on the membrane structure (for a recent review, see [29]). In this section, we briefly describe this last family of probes since they are very useful for studying lipid segregation.

In 1979, Gregorio Weber synthesized an exquisite polarity-sensitive probe named Prodan (6-propionyl-2-(dimethylamino)naphthalene, [30]). Figure 2 shows the structure of Laurdan, a derivative of Prodan that is widely used to explore the organization of biological membranes. The dipolar moment of Laurdan in the excited state changes due to an intramolecular charge transfer process [31] and thus the emission spectra of this probe is extremely sensitive to the polarity of its microenvironment. Due to its long hydrocarbonated chain, Laurdan is almost insoluble in water and efficiently partitions in membranes in such orientation that the fluorescent group is localized in the lipid-water interphase (Figure 2). When inserted in a membrane, the spectral position of Laurdan emission provides information on the local water content and thus on the local lipid packing. Since these properties are related to the local fluidity [32] this probe has been widely used in fluorescence spectroscopy and microscopy to explore fluidity changes and to identify lipid domains segregation in artificial and natural membranes (see for example, [33-35]).

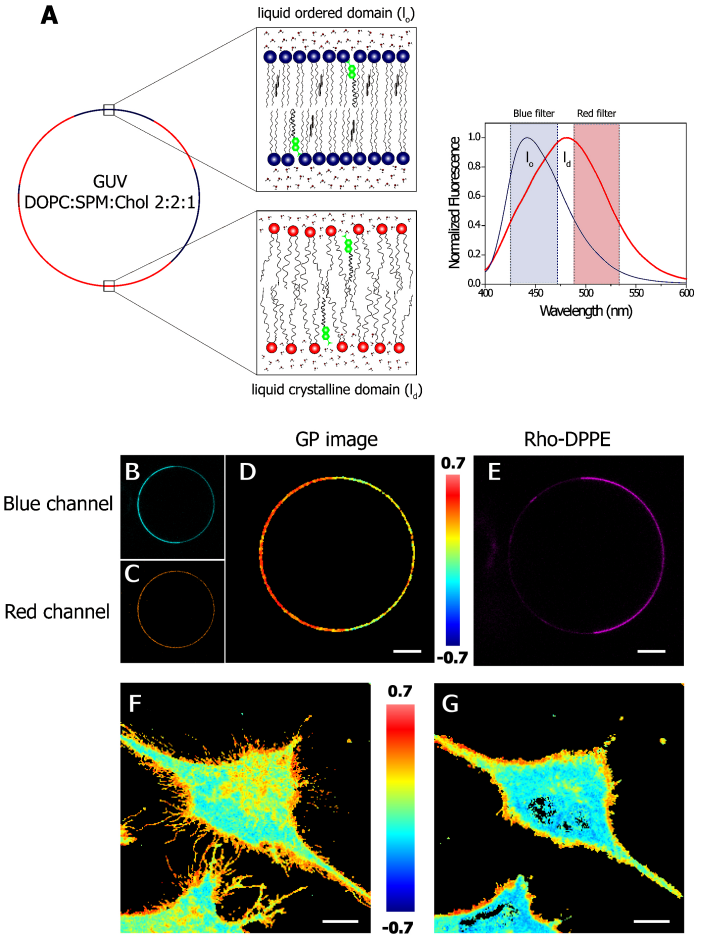


Figure 2. Measuring the fluidity of artificial and natural membranes. (A) Cartoon of a GUV presenting lateral separation between liquid crystalline ($l_d$) and liquid ordered ($l_o$) domains stained with Laurdan. The fluorophore locates at the lipid-water interface where it can sense the level of water penetration in the lipid bilayer. The emission spectrum of molecules inserted in the fluid phase (red) is red-shifted with respect to those located in liquid ordered domains (blue). (B-C) A GUV with a raft-like composition labelled with C- Laurdan was imaged in a confocal microscope using 2 different detectors set to collect fluorescence in the wavelength range showed in A (blue and red channel, respectively). (D) GP function is applied pixel by pixel to obtain the GP image of the GUV. High GP values reflect blue shifted emission corresponding to gel or liquid ordered domains and low GP reflect red shifted emission corresponding to liquid crystalline domains. (E) Image of the same GUV labelled with Rho-DPPE, a probe that is excluded from ordered domains. (F-G) GP images of a *Xenopus laevis* melanocyte stained with C-Laurdan and obtained at 2 different z-planes: close to the glass substratum (F) and at a central plane of the cell (G).

To characterize the spectral position of Laurdan, Parasassi et al., [36] defined the generalized polarization (GP) as,

$$GP = \frac{I_{Blue} - I_{Red}}{I_{Blue} + I_{Red}} \quad (1)$$

where $I_{Blue}$ and $I_{Red}$ represent the intensities registered at the blue or red region of the emission fluorescence spectrum; usually at 440 and 490 nm, respectively.

GP is a function widely used to quantify the spectral behavior of Laurdan in artificial and natural membranes and can be correlated to the local lipid packing and membrane fluidity [32, 36]. High GP values are obtained when the probe spectrum is blue-shifted and indicate higher order in the bilayer and low water content.

This probe was initially used for the quantification of membrane fluidity in spectroscopy assays. Since these measurements lack spatial resolution the GP analysis was then extended to fluorescence microscopy.

## DETECTING LIPID DOMAINS IN BIOMEMBRANES USING FLUORESCENCE MICROSCOPY

The combination of polarity sensitive fluorescent dyes such as Laurdan (providing information on the water content in the bilayer) with fluorescence microscopy (providing spatial resolution) allowed the visualization of lipid packing with submicron resolution in natural and artificial biological membranes. [37-40]

To perform GP-imaging experiments, the fluorescence intensity should be split in two different detectors with the emission filters set to collect the red and blue-side of the emission spectra as showed in Figure 2A. Then, GP values at every pixel of the image are calculated by applying Equation 1; further details regarding the instrumental corrections required for these microscopy measurements can be found elsewhere. [41]

This methodology has been widely applied in biophysical studies to explore the membrane organization of GUVs [23, 26, 41-45], changes in fluidity due to the interaction of specific proteins with the membrane [46,47] or changes in membrane packing due to the removal of cholesterol. [26, 48,49]

With the appropriate settings it is also possible to map lipid organization in 3 dimensions. [50-52]

Laurdan imaging normally requires the use of two-photon excitation microscopes to minimize photobleaching of the dye [41] constituting an important limitation since these microscopes are expensive and therefore not accessible to every research lab. Therefore, other environmentally sensitive probes such as di-4-ANEPPDHQ [53] and C-laurdan [54] were synthesized for confocal (one photon) studies. In the particular case of C-laurdan, we could observe that this probe can be used in combination with a conventional confocal microscope to image lipid organization in either artificial or natural membranes. [43]

Figure 2B-E shows an example of GP imaging in GUVs of a 'raft-forming' lipid mixture [55] composed of DOPC:SPM:Chol 2:2:1 at 25ºC. In this particular case, GUVs were labeled with C-Laurdan and Rhodamine-DPPE, this last probe only partitions into the liquid-crystalline phase [56]. The GP image of this particular GUV clearly shows the coexistence of two regions with different GP values; the high-GP regions can be assigned to "liquid-ordered" ($l_o$) domains mainly composed of sphingolipids and part of the cholesterol while the low-GP regions correspond to "liquid-disordered" ($l_d$) domains enriched in DOPC. [57] It is important to mention that GP imaging alone cannot be used to univocally assign the order of a lipid phase and other techniques such as deuterium NMR provide that information. [58] The aim of GP imaging is to distinguish phases with small differences in water content that can be correlated to its physical properties.

## IMAGING MEMBRANES FLUIDITY IN CELLS

GP-imaging technology initially developed for artificial lipid systems have been extended to the study of membrane organization in cells (see for example, [26, 50, 59-62]).

Figure 2F-G shows representative C-Laurdan GP images of cells. Usually, the GP values at the plasma membrane are higher than those measured at the internal membranes indicative of a lower fluidity of the plasma membrane when compared with the internal membranes. [43, 47, 61] While it is exceptional to observe large lipid domains at the plasma membrane [40], specific regions such as filopodia may present different fluidities (Figure 2F). This observation agrees well with that of Gaus et al., [40] who observed that filopodia present a higher lipid order.

GP imaging experiments are limited by the optical resolution of the microscope (~200 nm, [63]). As we mentioned before, rafts are small (20-300 nm) and highly mobile structures therefore even if Laurdan has the sensitivity to distinguish domains with different fluidity, the spatial resolution of the microscope is not enough for resolving rafts.

## DYNAMIC MEASUREMENTS

Dynamic GP imaging constituted a great innovation since it allowed observing dynamical membrane structures with sub-diffraction sizes. This new technique was introduced by Celli et al., [64, 65] and combines the GP methodology with fluorescence correlation spectroscopy (FCS).

FCS methods (e.g., [66-72]) are based on the analysis of intensity fluctuations caused by fluorescently labeled molecules moving through the small observation volume of a confocal or two-photon excitation microscope. Since the temporal window of these fluctuations is given by the processes determining the mobility of the molecules and their photophysics, this technique has been extensively applied to study diffusion, transport, chemical reactions, etc. (reviewed in [73]).

The analysis of fluorescence intensity fluctuations also provides the mean number of fluorescent molecules in the observation volume and thus it gives information regarding the local concentration of these molecules (see for example, [74, 75]).

The combined Laurdan GP/FCS methodology (Dynamic GP) consists on focusing the excitation laser at a position on the membrane and registering with a microsecond to millisecond time resolution the intensity fluctuations of Laurdan fluorescence in 2 different detection channels (Ch1 and Ch2 in Fig. 3) set as described for the static GP measurements. The intensity traces (i.e. intensity vs. time) obtained for each channel are processed using the GP function (Equation 1) to obtain GP traces containing the information of GP fluctuations at that particular position. Figure 3 schematically shows the output of these experiments.

This methodology was successfully used in GUVs to characterize the formation of sub-micrometric lipid domains in binary lipid mixtures by detecting GP fluctuations at the transition temperature [64, 65]. Very recently, the dynamic GP technique was applied to live cells (erythrocytes and CHO cells) [76]. Interestingly, static GP imaging in these cell lines showed a homogeneous GP value at the plasma membrane [77, 78] however the

combined approach gave a completely different perspective of membrane organization. These authors were able to detect the presence of diffusing structures with high GP values and sizes going from 20 to 300 nm (Figure 3C-D). The small size and characteristic high lipid packing of these micro-domains have the properties proposed for lipid rafts.

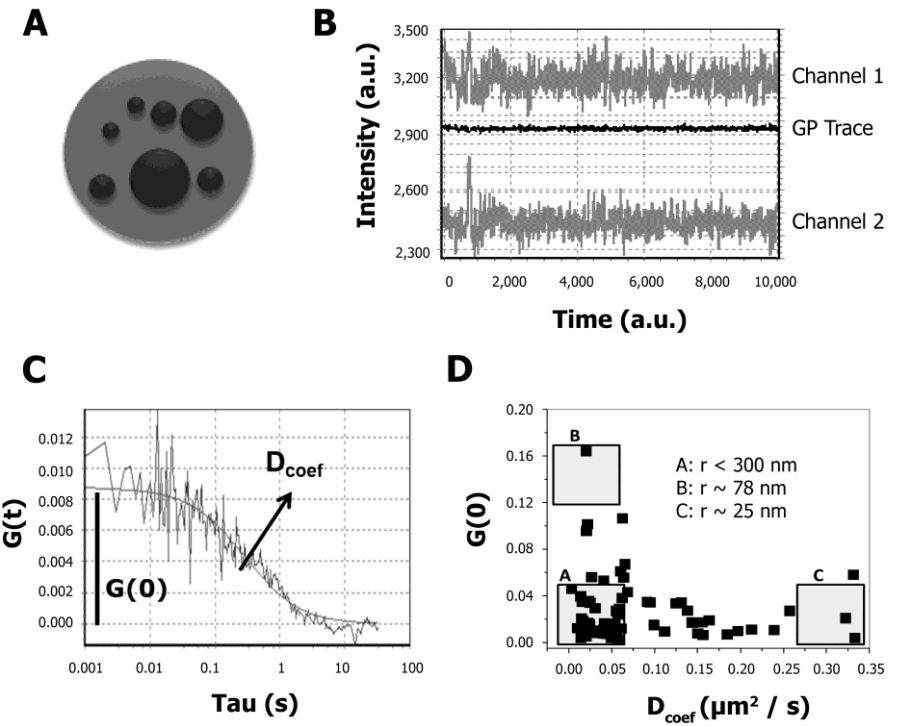


Figure 3. Dynamic Laurdan GP data acquisition and analysis. (A) 2D schematic representation of the observation volume of a 2-photon excitation microscope (large circle) focusing on a region of a membrane containing small, ordered nanodomains (small circles). Laurdan molecules distribute homogenously in the membrane but its emission spectra change depending on the local packing of the membrane. (B) The intensity of Laurdan molecules moving in and out of the volume is collected in two detectors set as described in the text (blue and red channels) and a GP trace is calculated from these data using Eq. 1. The fluctuations of this trace are not directly related to the diffusion of Laurdan molecules but to the changes in GP due to any structures with different GP value moving in and out of the volume. (C) The dynamic information hidden in the data is obtained by calculating the autocorrelation function that compares the GP value at time t, GP(t), with that at a time shifted by an amount $\tau$ (the lagtime), GP(t+$\tau$) as a function of $\tau$, i.e.,

$$G(\tau) = \frac{\langle GP(t) \cdot GP(t+\tau) \rangle}{\langle GP(t) \rangle^2} - 1$$

where the angled brackets indicate time averaged values.

These data are fitted with a passive diffusion model (continuous line) to obtain the diffusion coefficient ($D_{coeff}$) and the value of G(0) which is inversely proportional to the number of molecules in the observation volume [83]. (D) Several GP autocorrelations were analyzed from erythrocytes membranes labeled with Laurdan. The G(0) and $D_{coeff}$ from different GP autocorrelations are plotted in a fluctuation map which provides information on the size and abundance of nanodomains.

## CONCLUSION

The view of the structure and function of biological membranes has changed dramatically in recent years but still some topics are matter of debate. While it is widely accepted that cellular membranes are well-organized, dynamical structures, the existence of sphingolipid-enriched membrane rafts are still a controversial issue. This fact reflects the necessity of improved experimental techniques for raft detection.

In this article we reviewed approaches based on fluorescence microscopy that we believe may contribute to further characterize these sphingolipid-enriched domains in cell membranes during both physiological and pathological processes. Recently, two new approaches using Laurdan fluorescence have been developed.

GP function can be considered as a ratiometric parameter since it only evaluates fluorescence in two spectral windows. New confocal microscopes with the capability of acquisition of the whole emission spectra at every pixel of an image allow studying with high precision subtle spectral changes of Laurdan. This method is particularly useful to study dynamical changes in fluidity. [79] The second approach combines Laurdan and fluorescence lifetime imaging and allows the detection of subresolution-sized regions of different fluidities. [80-82]

In addition, new super-resolution techniques have been applied to the study of membrane organization (for a recent review see [83]). The application of these methods to raft research also looks extremely promising for the understanding of the biological role of sphingolipid-enriched domains *in vivo*.

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*Chapter 2*

# POTENTIAL ROLE FOR CERAMIDES IN NEURODEGENERATIVE DISEASES

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## ABSTRACT

Sphingolipids (SLs) are especially important in the central nervous system (CNS) where they are necessary structural component of membranes of brain cells or signaling molecules. Homeostasis of membrane sphingolipids in neurons and myelin is essential to preventing the loss of synaptic plasticity, cell death and neurodegeneration. Equilibrium of balance between specific SLs is essential for normal neuronal function. Even minor changes in the SLs balance can have dramatic effect on neurological and behavioral deficiencies. Over the past decade, it was found that relatively simple SLs, such as ceramide, sphingosine, sphingosine-1-phosphate and glucosylceramide play important roles in neuronal functions by regulating rates of neuronal growth, differentiation and death. Inducible dysfunction of the ceramide pathway, which is abundant in the brain as well as in peripheral organs,

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may account for neuronal disorders, behavioral symptoms, and further promote inflammation and oxidative stress.

Ceramides are the core constituent of most sphingolipids. They can be produced by hydrolysis of sphingomyelin (SM) via sphingomyelinases (SMases) or synthesized de novo from fatty acyl CoA and sphingosine. Ceramides are important second messenger molecules that regulate diverse cellular processes including cell growth, differentiation, and apoptosis. Ceramide levels in CNS also increase in response to aging and various age-related stress factors and are directly involved in apoptotic signaling in various neuronal cells, including neurons.

Because ceramides are so important as signalling components in the CNS, changes in brain ceramides levels due to their increased or decreased synthesis or metabolism may result in homeostatic dysregulation and ultimately neurodegeneration. This is extremely important because neurodegeneration is a characteristic component of all dementias.

In our review we discuss the possible sources of ceramides in CNS (1); summarize data about significant alteration in levels of ceramides in brain cells during development of different neurodegenerative disease such as Alzheimer's disease, Parkinson disease, multiple sclerosis, cerebral ischemia, Gaucher's, Farber's diseases, and etc. (2); present information about recent preclinical and clinical trials of therapies targeting ceramide pathway in brain.

Still it is currently unknown if ceramides are associated with CNS diseases through a direct or indirect mechanism. However, it is important to further study and confirm the role of these lipids in CNS (neurodegenerative) diseases as this would suggest possible modifiable risk factors that may serve as targets for strategies of prevention.

## ABBREVIATIONS

A[beta]--amyloid beta peptide,
AD - Alzheimer's Disease,
APP - amyloid precursor protein,
PD - Parkinson, disease,
HD - Huntington disease,
MS - multiple sclerosis,
Cer - ceramide,
CerS - synthase,
CSF - cerebrospinal fluid,
CNS - central nervous system,

DHCer - dihydroceramide,
DHSM - dihydrosphingomyelin,
SM - sphingomyelin,
SLs - Sphingolipids,
SMase - sphingomyelinase,
CERK - ceramide kinase,
ER - endoplasmic reticulum,
TNF–α - tumor necrosis factor-α,
IL–1β - interleukin-1β,
FAS L - FAS ligands.

## INTRODUCTION

Neurodegenerative diseases encompass devastating disorders of the central nervous system (CNS). Clinically these diseases are characterized by dementia, disordered movement, behavioral and psychiatric disturbance [1-3]. Pathologically they are characterized by inflammation [4], axonal degeneration [5], neuronal apoptosis [6] and demyelination for multiple sclerosis [7]. Genetic mutations, inflammation, environmental factors and activation of non-neuronal cells are predisposing influences. In some neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington disease (HD) aggregated proteins contribute to the neuronal pathogenesis [8-10]. In HIV-associated dementia (HAD) viral products are responsible for neuronal death [11], while in multiple sclerosis (MS) autoimmune mechanisms accompany the demyelination [7].

A shared biochemical cascades of events play crucial role in neuronal damage and have great importance for understanding of neuropathogenesis and potential for treatment of these diseases. Increased oxidative stress and activation of signaling pathway: the sphingomyelin-ceramide pathway are central between these cascades [12-14].

The SM pathway is triggered by endogenous and exogenous stimulants including tumor necrosis factor-α (TNF–α), interleukin-1β (IL–1β) [15], FAS ligands (FAS L) [16], glucocorticoids [17], anticancer drugs [18], ultraviolet [19] and ionizing radiation [20]. The ceramide serves as a second messenger of diverse signaling pathway in cell behaviors from proliferation and differentiation to cell arrest and apoposis [21, 22].

Ceramides are the core constituent of most sphingolipids. They can be produced by hydrolysis of sphingomyelin (SM) via sphingomyelinases (SMases) or synthesized de novo from fatty acyl CoA and sphingosine. There is increasing evidence that ceramides play a decisive role in the neuronal function due to their regulatory effects on the growth rate, differentiation and death of cells in the CNS [23]. Imbalance in content of various classes of sphingolipids results in neuronal dysfunction and apoptosis of brain cells. Because ceramides are so important as signalling components in the CNS, changes in brain ceramides levels due to their increased or decreased synthesis or metabolism may result in homeostatic dysregulation and ultimately neurodegeneration. This is extremely important because neurodegeneration is a characteristic component of all dementias.

Thus, induction of the sphingomyelin cascade cycle resulting in accumulation of the proapoptotic agent ceramide may be considered as a novel mechanism of the development of neurodegenerative diseases. This may by considered as a prerequisite for novel approaches to therapy of this diseases by using drugs of a new generation that inhibit SMase activity leading to accumulation of ceramides in brain cells [24-26].

## CERAMIDES

Ceramides are the core structure of a class of complex lipid called sphingolipids, ubiquitous components of eukaryotic cell membranes. They are a family of lipid molecules that consist of sphingoid long-chain base linked to an acyl chain via an amide bond. Ceramides differ from each other by length, hydroxylation and saturation of both the sphingoid base and fatty acid moieties. The fatty acid components of ceramides vary widly in composition. Their acyl chain lengths range from 14 to 26 carbon atoms although the most common fatty acids are palmitic (C16:0) and stearic (C18:0) non-hydroxy fatty acids. The fatty acids are commonly saturated or mono-unsaturated [27]. Small changes in the molecular structure of ceramide moiety can regulate its biological function. Naturally occurring long-ceramides are eminently hydrophobic even compared to other lipid species. This hydrophobicity of ceramides justifies the need for a ceramide transfer protein (CERT) in cells [28]. Free ceramides are molecules known to exert a wide range of biological functions in many of the most critical cellular events, including growth, differentiation, apoptosis and oncogenesis [29].

Recent advances in the development of new analytical methods for rapid and sensitive qualitative analysis of ceramide species in biological samples with using of electrospray ionization tandem mass spectrometry have provide a set of effective and essential tools for the analysis of ceramide content in brain tissues [30, 31]. Such analyses are necessary in order to delineate the mechanisms by which the various ceramides contribute to or protect from neurodegenerative processes. Furthermore, the analysis of genes that code for proteins involved in ceramide metabolism in neural cells clears the molecular mechanisms by which ceramide levels are controlled [31].

## CERAMIDE GENERATION

Ceramides can be produced in cells either via the *de novo* synthesis or via hydrolysis of complex sphingolipids. The catabolic enzymes produce ceramide within a few minutes whereas the *de novo* synthesis produces ceramide in several hours [32, 33]. In animal cells ceramide is synthesized *de novo* on the endoplasmic reticulum [27, 34] and in mitochondria [35]. Mutations in genes of enzymes responsible for *de novo* synthesis of ceramides underlie a neurodegenerative disorders of the peripheral nervous system [36]. Neo-synthesized ceramides subsequently traffic from the luminal face of the ER to the Golgi compartment where different polar heads are incorporated into the ceramide molecule to form complex sphingolipids.

Beside the *de novo* pathway significant contribution to intracellular ceramide levels occur also through hydrolysis of complex sphingolipids by activation of different hydrolases. Ceramides derived from sphingomyelin (SM) catabolism require the activation of sphingomyelinases (SMase), which hydrolyze the phosphodiester bond of SM yielding water soluble phosphorylcholine and ceramide [37, 38].

These enzymes are characterized by certain pH optimum (acidic, alkaline, and neutral sphingomyelinases, (aSMase, alkSMase, and nSmase, respectively), cell localization and metal-ion dependency. Although aSMase is a Zn2+-dependent enzyme preferentially localized in lysosomes [38], its secretory form is also known [37]. The structure of alkSMase differs from other SMases; although this enzyme belongs to the family of nucleotide pyrophosphates /phosphodiesterases its enzymatic properties coincide with those of other enzymes cleaving sphingomyelin to ceramide [39]. aSMase is especially active in the intestinal mucosa and the bile, where it cleaves dietary

sphingomyelins [40]. aSMase is active and highly expressed in brain cells uniformly throughout brain development [40].

The family of nSMases characterized by the pH optimum of 7.4 includes three enzymes differed by their cellular localization and ion dependency. nSMase1 is a Mg2+-dependent enzyme of 47.5 kDa localized in the endoplasmic reticulum [41]. nSMase2 is located in the Golgi apparatus [37, 42] and this enzyme may be translocated to the perinuclear space in response to the antioxidant effect of glutathione, while during oxidative stress nSMase1 is translocated to the plasma membrane [42]. Phosphorylation has been regarded as an important mechanism for nSMase 2 activity [37]. nSMase3 is also detected in the Golgi apparatus, endoplasmic reticulum, and plasma membrane [43]. nSMases as well as aSMases are actively expressed in brain cells; especially active expression of nSMases is observed during neuronal development.

A functional role of nSMase1 and nSMase3 remains poorly understood, while there is evidence that nSMase2 is involved in cell signaling and pathogenesis of some neurological diseases [37].

Now it is firmly recognized that nSMase2 is involved in such neurological diseases as senile dementia, AD, AIDS dementia, Huntington's disease, multiple sclerosis, lateral amyotrophic sclerosis (progressive muscular atrophy), strokes, etc [37]. Pathogenesis of all these diseases shares such common features as increased neuronal apoptosis or oxidative stress.

Conversion of a ceramide into sphingomyelin involves phosphatidyl-choline transferase; this enzyme catalyzes transfer of the phosphocholine group of phosphatidylcholine onto the ceramide. Such type of phosphatidylcholine transferase is also known as sphingomyelin synthase (SMS). Two sphingomyelin synthases are known, SMS1 and SMS2. In humans SMS1 is localized in the Golgi apparatus, while SMS2 initially appears on the plasma membrane [44, 45].

Besides involvement into sphingomyelin biosynthesis ceramide may be further metabolized in sphingosine and fatty acids in the reaction catalyzed by ceramidases. Like SMases the ceramidases also differ by pH optimum of their catalytic activity and cell localization. Five ceramidases are known to date [46]. Acid ceramidase is preferentially localized in lysosomal compartments; neutral ceramidase is preferentially located in the plasma membrane, while three alkaline ceramidases have been found in the Golgi apparatus and the plasma membrane.

Sphingosine phosphorylation by sphingosine kinase results in formation of sphingosine-1-phosphate (S1P), a antiapoptotic agent [47].

Ceramide and S1P that exert effect of opposite nature in their regulation of apoptosis, differentiation, proliferation and cell migration. The concentration of ceramide and S1P is counter-balanced by enzymes that convert one lipid to the other and their levels balance between cell viability and cell death. However, this is not the only way the cell can balance to ensure tissue homeostasis. Ceramides can also be phosphorylated by the enzyme ceramide kinase (CERK) to form ceramide-1phosphate (Cer1P) [48]. Cer1P inhibits apoptosis and can induce cell survival. CERK was fiest observed in brain synaptic vesicle and highly expressed in brain, heart, skeletal muscles and liver [48]. Localization of enzyme at synaptic- vesicles suggests a possible role for CERK in neurotransmitter release.

## CERAMIDES IN APOPTOSIS

Neurodegenerative diseases such as Parkinson's, Alzheimer's, Huntington's and Prion diseases are all characterized by a massive loss of specific population of neurons or damage to neuronal transmission. Apoptosis is an essential process responsible for neuronal damages [49]. The role of ceramide in apoptosis is very complex. An increase of ceramide levels leads to cell death [49], but depletion of ceramide can reduce the progression of apoptosis [50]. However, ceramide is very important for proper function of the CNS. This outlines the importance for neuronal cells to maintain a ceramide balance by tight regulation of sphingolipid signaling networks.

Ceramide can induce apoptosis via different ways and different intracellular organelles.

SM hydrolysis by neutral and/or acid SMases is very important pathway for generation of pro-apoptotic ceramides [51]. SM hydrolysis generates a rapid increase of ceramide. However, the *de novo* synthesis of ceramides is relevant in generating of a signaling pool of ceramide leading to cellular apoptosis [52]. The ceramide pathway *de novo* requires multiple enzymatic steps and period of several hours.

SMase activation occurs in response to stimulation of cell surface receptors of the tumor necrosis factors (TNF) upon the binding with specific ligands as TNF alpha [53]. TNF alpha is one of the main factors affectiong neurodegeneration and contributes to the neuronal pathogenesis [54].

The ceramide generated by nSMase leads to the activation of ceramide-activated protein kinase (CAPK) [55] and ceramide-activated protein phosphatases (CAPPs) [56]. CAPK is involved in mitigen-activated protein

kinase (MAPK) cascade that induce the activation of extracellular-signal regulated kinases (ERK). ERK cascade leads to cell cycle arrest and cell death. CAPPs mediate the effect of ceramide through dephosphorylation and inactivation of several substrates, such as retinoblastoma gene product (RB) [57], Bcl-2 and Akt [58] and through downregulation of the transcription factor c-Myc [59] and c-Jun [60].

Ceramide produced by aSMase activates the aspartyl protease cathepsin D [61] that can subsequently cleave the pro-apoptotic Bcl-2 family member Bid. Activation of Bid induces cytochrome c release from mitochondria and activation of caspase-9 and-3, leading to apoptotic cell death [62].

There are numerous indications of links between ceramide elevation and mitochondrial apoptosis. In cortical neurons ceramide treatment leads to Akt dephosphorylation, mitochondrial depolarization and permeabilization [63], cytochrome release [64] and activaton caspase-3 and caspase-9 [65]. Ceramide directly affects the generation of reactive oxygen species (ROS) from mitochondria [66].

## CERAMIDES IN ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common cause of senile dementia in elderly people. A significant proportion of population over the age 60 years now suffers from AD. It is generally believed, that the toxic effect of amyloid beta peptide (Aβ), the component of amyloid plaques, and cytokine-mediated inflammatory processes play the key role in AD [67]. Aβ deposits and maturation of senile plaques induce numerous molecular changes resulting in progressive dysfunction and death of neural cells via apoptosis or necrosis [67]. However, molecular mechanisms of etiology and pathogenesis of AD remain unknown. During the last decade studies on mechanisms of AD are focused on brain lipids as the main components of cell membrane involved in processing and aggregation of Aβ and transduction of the cytotoxic signal induced by both Aβ and the proinflammatory cytokine TNF-α [67,68].

Experiments on brain cells provided direct evidence that SMase, the enzyme involved in sphingolipid metabolism, plays a certain role in Aβ cytotoxicity [69, 70-77].

Experiments on primary neuronal cells have shown that treatment with fibrillar Aβ causes nSMase expression and the increase in ceramide content [73]. Inhibition of this enzyme decreased cell death thus suggesting nSMase

requirement for Aβ cytotoxicity [73]. Inhibition of nSMase by glutathione also decreased dendrocyte death [71]. These results were also confirmed by studies of the toxic effect of A25-35 on glial and endothelial cells. As in the former case addition of S-nitrosoglutathione caused a neuroprotective effect [72].

It should be noted that certain discrepancy does exist in results on the role of SMases responsible for Aβ-induced cell death. For example, the effect of Aβ on mouse dendritic cells resulted in activation of aSMase, while inhibition of this enzyme determined resistance of these cells to Aβ-induced apoptosis [74]. A soluble Aβ oligomer caused activation of both nSMase and aSMase. Specific inhibition and knockdown of each enzyme provided cell resistance to Aβ-induced apoptosis [73]. These results suggest involvement of both types of SMase in realization of Aβ-induced apoptosis of nerve cells. It was demonstrated that intracerebral administration of Aβ or TNF-α to rats resulted in nSMase activation. This process was more pronounced in hippocampus than in cortex and cerebellum [77, 78]. However, postmortem analysis of brain of AD patients revealed activation of only aSMase [79]. Expression analysis of genes encoding aSMase and nSMase2 revealed their sharp activation in the brain of patients with both AD and other neuropathologies [31].

Since SMase activation was investigated in various cell lines, during actions of various forms of Aβ, in animal experiments and in studies of human postmortem brain preparations, existence of certain discrepancies in results obtained in different systems is not surprising. There is no reasonable transgenic animal model, which would completely mimicked the pathophysiological process of AD and still remains unclear which type of Aβ is responsible for cell death and loss of cognitive impairments in humans.

Although local concentrations of fibrillar Aβ found in the brain of AD patients may differ from Aβ concentrations in primary neurons used in experiments, the data on interrelationship between SMase activation, ceramide accumulation and subsequent death of neurons and oligodendrocytes suggest that SMase may be a perspective target for drugs preventing neurodegenerative impairments in AD.

## THE MECHANISM UNDERLYING INVOLVEMENT OF CERAMIDE IN THE PATHOGENESIS OF AD

Various factors such as cytokines, growth factors, hormones, oxidative stress, and radiation activate SMase in various tissues thus causing ceramide

accumulation in them [15-20, 80]. Mechanisms responsible for SMase activation in neurons and activated glial cells followed by significant activation of ceramide levels in them still require better understanding. This is very important because knowledge of glial cell activation is associated with loss of neurons during the development of certain neurodegenerative diseases including AD. Astrocytes, the main representative of glial cells, activated by Aβ1 - 42 in combination with 10 ng/ml IL-1β were used in studies of mechanisms of their toxic effect on human primary neurons [70, 81]. These studies demonstrated a sharp activation of nSMase and ceramide accumulation in neurons during their death induced by astroglia activation. Activation of neuronal nSMase is determined by NO generated by activated astroglia. At the same time it was found, that in activated astrocytes nSMase could induce mRNA expression of inducible nitric oxide synthase (iNOS). It was also found that expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6 in the activated astroglia depended on nSMase. Involvement of A[beta] in ceramide generation was demonstrated by the fact that only Aβ1--42 but not its reverse form (Aβ42--1) induced SMase activation and ceramide generation in neurons [82]. In addition, the fibrillar form of this peptide exhibited the highest efficiency in ceramide generation compared with its soluble form [82]. Aβ also induced apoptosis in oligodendrocytes. As in the case of neurons this was accompanied by the increase in the ceramide level [71], caspase-8 activation and DNA fragmentation [71]. Addition of exogenous ceramide or bacterial SMase increasing the ceramide level to the oligodendrocyte cell culture caused death of cultivated cells [71, 74]. An SMase inhibitor (3-methyl-sphingomyelin) effectively protected oligodendrocytes against Aβ [71].

Expression of iNOS is very important for manifestation of the toxic effect of Aβ on brain cells. The mechanism of activation of the signal system NO and the development of oxidative stress induced by the Aβ effect on CNS cells was demonstrated using oligodendrocytes [75]. This process was accompanied by activation of the metabolic pathway, which involves nSMase and cause ceramide accumulation [83]. However, it was demonstrated that acting independently, neither ceramide nor Aβ] caused iNOS expression. The latter required accumulation of TNF-α, which induced this process, while Aβ was only able to activate nSMase. This fact is important for understanding the mechanism of AD development, in which proinflammatory components plays an important role [75].

Nerve growth factor increased ceramide level in glioma T9 cells and hippocampal neurons via activation of nSMase [84]. In some cases stress

activated signals caused activation of aSMase in hippocampal neurons [85]. Since various SMases are localized in different cell compartments, this means that different stimuli cause generation of different ceramide pools [22]. The mitochondrial ceramide pool is especially important for Aβ-induced apoptosis [86].

Animal experiments have shown that microinjection of Aβ1-42 into cortex of C57/BL6 mice caused a 3-fold increase in cortex ceramide induced by nSMase, while activity of aSMase under these conditions remained unchanged [70]. Intracerebral administration of Aβ to rats caused preferential accumulation of ceramide in hippocampus [77].

This is consistent with increased levels of ceramides found in the brain and cerebrospinal fluid of AD patients compared with patients with other neurological pathologies and ceramide accumulation was detected in AD patients during early stages of this disease [82, 87, 88]. Other authors found decreased levels of ceramide in the white matter of the middle frontal gyrus in AD patients compared with corresponding control [82]. These differences are obviously determined by a stage of this disease, because recently it has been demonstrated that the ceramide level was higher on the early stage of dementia, while the later stages were characterized by decreased ceramide levels in brain structures [82].

Analysis of six ceramide species differed by the length of their fatty acids revealed increased levels of Cer16, Cer,18, Cer20, and Cer24 in the brain not only of AD patients but also in patients with all other brain impairments studied. The highest ceramide level was found in patients characterized by combination of AD and other pathologies [31]. The most definitive results were obtained during expression analysis of gene involved in control of synthesis, metabolism, and degradation of ceramides in the development and course of AD. There were opposite activation of genes responsible for ceramide content in brain structures in specific forms of this disease compared with control [89]. It was firmly recognized that the early stage of this disease was characterized by increased synthesis of enzymes involved in ceramide synthesis [89] especially containing long chain fatty acids C22:0 and C24:0, while synthesis of glucosylceramide decreased. In other words levels of various types of ceramides may undergo opposite changes.

Some studies revealed association of the sphingomyelin cycle signaling system with oxidative stress [37, 83]. It was found that reactive oxygen species directly influenced SMase or other enzymes involved in regulation of SMase activity. This may potentiate the toxic effect of cytokines and Aβoν the brain cells during combined action of these compounds. It should be noted that the

natural antioxidant glutathione inhibits SMase activity. A decrease in the glutathione level causes activation of this enzyme and accumulation of ceramides in oligodendrocytes followed by their death [71]. Successful employment of antioxidant therapy in clinical practice confirms effective inhibition of processes related to activation of oxidative systems in the development of AD. However, it still remains unclear whether this is accompanied by inhibition of the sphingomyelin cycle.

Thus, induction of the sphingomyelin cascade cycle resulting in accumulation of the proapoptotic agent ceramide may be considered as a novel mechanism of the development of AD. This may by considered as a prerequisite for novel approaches to AD therapy by using drugs of a new generation that inhibit SMase activity [90-92].

## BLOOD CERAMIDES IN AD

Analyzing various blood biomarkers in various neuropathologies a reasonable question arises: does direct correlation between changes in blood and brain processes exist? It is possible that blood changes may be associated with the brain via the circulation system and selective permeability the blood brain barrier? Thus, it is important to determine the pattern of changes in the level of ceramides during the development of AD.

At the early stage of AD the blood ceramide content decreased, while brain and CSF concentrations increased [93-95]. These changes correlated with cognitive impairments in AD patients. However, limited data on this subject hampers the use of blood ceramides in AD diagnostics, and further studies, which would include correlation analysis between blood and CSF ceramide content during the whole period of AD are clearly needed. Some researchers believe, that correlation between changes of blood and CSF sphingolipids in AD patients implies applicability of sphingolipids as the AD biomarkers (especially at the early stage of this disease) and putative targets for generation novel drugs [94, 95].

Recently, the interrelationship between changes of plasma sphingolipids and cognitive impairments is intensively studied in AD patients for predicting of the rate of the development of dementia by results of sphingolipid analysis [93-95]. This task may be solved due to the development of MS and its successful use in lipidology.

M.M. Mielke et al., [93, 95] studied the content of ceramides (Cer), dihydroceramides (DHCer), sphingomyelins (SM) and dihydrosphingomyelins

(DHSM) in blood plasma of 120 patients with dementia of the Alzheimer's type and dementias associated with other neuropathologies. During the period of more than two years of observation authors found an increase in Cer and DHCer associated with rapidly progressive dementia. In patients characterized by increased levels of SM and DHSM and the ratio SM/Cer and DHSM/DHCer slowly progressive dementia. These authors also demonstrated that changes in cholesterol and triglyceride levels were mot associated with the rate of dementia. This suggests that the increase in the ratio SM/Cer and DHSM/DHCer in blood of AD patients may be a predictive marker for the rate of the development of this disease.

Similar results have been obtained in [96] during MS-analysis of blood plasma lipids in 26 AD patients and 26 elderly patients with normal cognitive functions. Among 33 sphingomyelins, the content of eight molecular species containing fatty acid aliphatic chains of 22 and 24 carbon atoms was significantly lower in AD patients compared with control. At the same time, the plasma levels of two ceramides (16:0 and 21:0) were significantly higher in AD patients, while 5 ceramides increased insignificantly. The ratio of ceramides to sphingomyelins containing identical fatty acids sharply differed in AD patients compared with normal controls. These changes reflected both impairments of cognitive functions in AD patients and in genotype (during diagnostics particular genotype was determined by apolipoprotein E4) [96].

In addition, capacities of the sphingolipid assay for evaluation of AD progression would be useful for monitoring of effectiveness of treatment of individual patients by certain drugs. The latter is especially important when patients are treated by novel drugs or novel drugs are under preclinical or early clinical trials.

## CERAMIDES IN PARKINSON'S DISEASE

Parkinson's disease (PD) is a common neurodegenerative disease which affects over 1% of people over the age of 65 years [97]. Clinical manifestation include bradykinesia, rigidity, tremor and postural instability. PD is characterized by deposition of brain alpha-synuclein, a lipid-binding protein, and dopamine neuron degeneration. Additionally, typical PD cases have intracellular proteinaceous inclusions called Lewy bodies and Lewy Neurites in the brainstem and cortical areas [98].

It has been suggested that perturbation in ceramide metabolism [99] may contribute to alpha-synuclein deposition and the formation of Lewy bodies

[100]. Mutations in the beta-glucosidase gene (GBA) coding glucocerebrosidase, which breaks down glucosylceramide into glucose and ceramide, are the most common genetic risk factors for sporadic PD [101].

Many others different cellular processes have been implicated in PD, including generation of reactive oxygen species, endoplasmic reticulum (ER) stress, compromised mitochondrial function, diminished function of the ubiquitin proteasome system and protein aggregation [102]. Additionally, inflammation and activated microglia have been generally implicated in PD pathology [13, 104] and increased levels of pro-inflammatory cytokines such as TNF, IL-1β and IL-6, have been observed in the cerebral spinal fluid (CSF) and striatum of PD patients relative to healthy age-matched controls [105]. Furthermore, gene polymorphisms in inflammatory genes (TNF-308 and IL-1β-511) have been associated with an increased risk of developing PD [106]. Dopamine neurons (DA), are acutely sensitive to TNF-induced toxicity [107]. Soluble TNF signals through the canonical transmembrane death receptor TNF receptor1 (TNFR1) to potently transduce inflammatory stimuli [108]. TNFR1 is constitutively expressed in DA neurons. However, TNFR1 can elicit signaling through numerous downstream effectors, including ceramide [109]. But identification of specific pathways required for TNF-induced cytotoxicity in DA neurons has not yet been forthcoming. Specifically, ceramide has been implicated in the cell death pathway activated by the death domain receptor ligands TNF and Fas-L [110]. Additionally, ceramide has been shown to activate apoptosis in primary cortical neurons [111] and in primary neuronal cultures from embryonic mesencephalic neurons [112], but its role as a critical downstream effector of TNF-induced apoptosis in DA neurons has not been fully delineated.

Martinez and coauthors report that TNF and ceramide exert dose-dependent cytotoxic effects on DA neuroblastoma cells and primary DA neurons [113]. Functionally, inhibitors of SMase activity which block sphingomyelin hydrolysis and ceramide generation attenuated TNF-induced cytotoxicity, decreases in phospho-Akt, increases in caspase 3 cleavage as well as mitochondrial membrane potential changes, and ER stress in DA cells. Ultimately, the mechanisms of TNF-induced cytotoxicity in DA cells culminated in and were found to be completely dependent on caspase signaling, suggesting a model in which ceramide/sphingolipid signaling cascades are key effectors of TNF-dependent apoptotic death in DA neurons.

Their data also revealed that TNF treatment not only activates sphingomyelinases (SMases) to produce ceramide but also leads to generation of several other atypical deoxy-sphingoid bases (DSBs) including

desoxymethylsphingosine (1-desoxyMeSo), deoxysphinganine (deoxySa), and desoxymethylsphinganine (1-desoxyMeSa); when added exogenously in vitro, some of these DSBs inhibit neurite outgrowth and are toxic to DA neurons. These findings suggest that multiple sphingolipid mediators may be responsible for mediating TNF neurotoxicity and identification of specific sphingolipid metabolites may reveal opportunities for drug development to delay or prevent DA neuron degeneration [113].

Dopaminergic neurons positively regulate neutral sphingomyelinase activity, thereby increasing ceramide levels, in response to oxidative stress. [114]. Ceramide has been shown to induce apoptosis in dopaminergic models [115]. This effect is neutralized by the non-pathological gene products of parkin [116] and alpha-synuclein [117].

Determination of ceramides species in plasma of PD patients has been shown that several species of ceramides, monohexosylceramides and lactosylceramides were elevated in PD patients versus controls and were highest among those PD patients who had cognitive impairment [118]. Post-mortem and *in vitro* PD studies have indirectly demonstrated that the activation of ceramide signaling may mediate the apoptosis in the substantia nigra [119]. It was shown that intracellular glucosylceramide levels control the formation of soluble toxic α–synuclein assemblies in neurons and in mouse and human brain, leading to neurodegeneration [120]. The elevation and formation of α–synuclein assemblies inhibits the lysosomal activity of normal glucocerebrosidase in neurons and PD brain, resulting in additional glucosylceramide accumulation and augmented α–synuclein oligomer formation, resulting in neurodegeneration[120].

It was hypothesized that changes in ceramides level may be involved in pathogenesis of PD. Identifying the regulatory mechanisms precipitating these changes may provide novel targets for future therapeutics.

## CERAMIDES IN MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the CNS in which oxidative stress plays a pathogenic role. Reactive oxygen species (ROS) generated by activated macrophages and microglial cells are thought to play a major role in damaging of myelin and oligodendrocytes in MS [121]. Mechanisms that might protect oligodendrocytes from oxidative stress are poorly understood. Recently it was

shown that oxidative stress kills human primary oligodendrocytes via the activation of n-SMase-ceramide pathway. It was demonstrated that H2O2 and ATZ-inhibitor of catalase, that increases intercellular concentration of H2O2, markedly induced the activation of n-SMase and cell death in oligodendrocytes [122]. However, antisense knockdown of n-SMase protected oligodendrocytes from H2O2- and ATZ-induced cell death. On the other hand, knockdown of acidic sphingomyelinase did not abrogate oligodendroglial cell death [122]. These results clearly implicate the central role of n-SMase in endangering oligodendrocytes to oxidative-stress-induced cell death. Mechanisms involved in ROS-mediated activation of N-SMase in primary oligodendrocytes are not known. There is evidence that ROS modulate both PKC [123] and tyrosine kinase activities or inhibit tyrosine phosphatase activities [124]. Therefore ROS may induce the activation of n-SMase by activating one or several protein kinases or inhibiting tyrosine phosphatase activities. Ceramide, a putative second messenger, produced from the degradation of sphingomyelin by sphingomyelinases is inducer of apoptosis in various cell types including glial, neuronal and oligodendroglial cells [125]. It means that various ROS-producing molecules and ROS itself induce the activation of N- sphingomyelinase and the production of ceramide in human primary oligodendrocytes.

Antioxidant treatment of patients with MS is more popular therapeutic approach.

Injection of antioxidants such as GSH to mice decreased activity of n-sphingomyelinase in mice liver [126]. The data obtained with animals support data described by Hannun and other authors in vitro [127]. There are many indications that GSH influences the SMase-ceramide pathway in apoptotic process. It was shown that GSH inhibits apoptosis in isolated thymocites treated by sphingosine and UV-irradiation [128].

While manipulation with nonenzymatic antioxidant system affect ceramide production, formation of this sphingolipid can also modulate oxidative stress. Exposure of cultured rat hepatocytes to exogenous bacterial SMase [129] and injection of ceramide to mice resulted in ROS formation in brain cells [130]. Moreover it was shown that injection of ceramide induced accumulation of TNF-α in mice brain. It means that generation of sphingomyelin product by exogenous TNF-α can induce endogenous accumulation of TNF-α which in its turn generates new portion of ceramide. [130].

A lipidomic profiling approach led to the identification of increased level of ceramide C16:0 and C24:0 in the cerebrospinal fluid from patients with

multiple sclerosis. Expose of cultured neurons to cerebrospinal fluid from patients with MS and micelles composed of these ceramide species induced oxidative damage [131].

These results indicate that attenuation of neutral sphingomyelinase may constitute a crucial role in preventing oligodendrial loss in demyelinating disorders in MS [132].

## CERAMIDE IN ISCHEMIA-ASSOCIATED NEURONAL DAMAGE

Ischemic stroke induced by a temporary or permanent reduction of local cerebral blood flow is the leading cause of death and disability in humans. Among the several molecular mechanisms that contribute to ischemia-induced brain damage, inflammation plays a crucial role [133, 134]. Recent studies suggest that astrocytes are involved in inflammation through release of cytotoxic agents in response to ischemia stimuli [135]. Ceramide has been identified as an important signaling molecule in inflammation. It was shown at the ischemia model with four-vessel occlusion and oxygen-glucose deprivation that astrocytes are involved in neuroinflammation through specific accumulation of ceramide in hippocampus astrocytes but not in neurons. After 10 min of ischemia followed by 30 min of reperfusion, a considerable level of ceramide was found in CA1, CA2 and CA3/dentate gyrus hippocampal areas primary in astrocytes but not in neurons. This data were obtained by immunohistochemical and immunofluorescence double-staining analysis [136]. Generation of ceramides in cerebral ischemia is induced by nSMase2 which is responsible for ceramide production in the neuronal membrane. It was clear shown that ceramide derived from SM hydrolysis by nSMase2 but not aSMase. nSMase2 is a phosphoprotein with five highly conserved serine residues (S173, S208, S289, S292, and S299) and its activity can be regulated by kinases and phosphatases in response to certain stresses [137]. Phosphorylation plays a crucial role in nSMase2 activity. It was found that p38MAPK is involved in nSmase2 activation in rat hippocampi following ischemia [138]. Real-time PCR detection of mRNA level of inflammatory cytokines such as IL-1β, IL-6 and TNF-α in ischemic rat brain indicated that the activation of nSMase2 could drive the generation and release of inflammatory cytokines. Therefore, p38MAPK, nSMase and ceramide signaling are closely associated with the upregulation of inflammatory factors

in rat hippocampi in response to ischemia [136]. It has also been reported that transient focal cerebral ischemia induces large increases in aSMase activity, ceramide levels and production of inflammatory cytokines in wild-type mice, but not in mice lacking aSMase. [139-141]. Reduction of ceramide levels by an nSMase2 inhibitor could attenuate mRNA levels of proinflammatory cytokines in the hippocampus following ischemia [142].

It is well known now that inflammation induced by ceramide in astrocytes is closely associated with neuronal damage following cerebral ischemia. These results indicate that the inhibition of ceramide production in astrocytes by targeting p38MAPK/nSMase2 signaling may represent a real approach for attenuating inflammatory responses and neuronal damage after cerebral ischemia.

## CERAMIDES IN STORAGE DISORDERS

All sphingolipids are synthesized from ceramides and hydrolyzed to ceramides. In addition to CDase and SMase, there are other hydrolytic enzymes which hydrolyze complex sphingolipids to ceramides. The brain is the organ mainly affected by accumulation of sphingolipids products due to its high metabolism of sphingolipids. Disorders of sphingolipids metabolism in lisosomes induced a family diseases identified as lysosomal storage diseases (LSDs). LSDs include Niemann-Pick disease (NP), Gaucher's disease, Farbe's disease and Krabbe's disease.

Niemann-Pick disease type A and B (NP) is characterized by the absence of aSMase resulting in defect of SM degradation. SM cannot convert to ceramide and it is alterated the ceramide-SM ratio [143, 144]. It means that signals cascades with participation of ceramide is not developed.

Gaucher's disease is caused by the absence of glucosylceramidase [145-148]. Deficiency of these enzyme causes neuronal storage of gangliosides leading to loss of neurons and their axons, resulting in cortical atrophy and white matter degeneration [149].

Farbe's disease is characterized by the deficient activity of aCDase and high levels of ceramides [150, 151]. The rate of ceramide synthesis in brain during development of Farbe's disease is normal but ceramide resulting from degradation of complex sphingolipids cannot be hydrolyzed and accumulated into lysosomal compartment [152]. The abnormal ceramide level in the brain results in neuronal dysfunction.

Krabbe's disease is caused by the deficiency of galactosylceramidase in lysosomas which remove galactose from galactoceramide derivatives [153]. Abnormal storage of galactosylceramide leads to apoptosis of myelin forming cells with a complete arrest of myelin formation and axonal degeneration.

## CONCLUSION

During two last decades much attention is paid to brain sphingolipids involved in pathogenesis of different type of neurodegenerative diseases. Studies of sphingolipid metabolism in brain structure of humans and experimental animals, in CSF, in plasma and serum of patients during the course of neurodegenerative diseases such as AD, PD, MS, cerebral ischemia and etc., have shown that ceramide plays a decisive role in the neuronal function due to regulation growth, differentiation and death of cells in the CNS. Activation of the sphingomyelinase-ceramide pathway via oxidative stress mechanism plays a cardinal role in apoptosis of neurons and oligodendrocytes. Accumulation of the proapoptotic agent ceramide may be considered as a novel mechanism for the development of different type of neurodegenerative diseases. This is a prerequisite for treatment of these diseases using novel drugs inhibiting SMase activity.

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*Chapter 3*

# INTERPLAY BETWEEN Aβ, CERAMIDES AND HYPERPHOSPHORYLATED TAU IN ALZHEIMER'S DISEASE

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## ABSTRACT

Alzheimer's disease (AD), the most common leading form of dementia in elderly people, is a chronic and progressive neurodegenerative disorder. The most frequently investigated neuropathological hallmarks of AD are extracellular deposits of neurotoxic amyloid β peptide (Aβ) and intracellular aggregates of

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hyperphosphorylated tau protein. Ceramides, the major molecules of sphingolipid metabolism, have been linked to AD susceptibility and pathogenesis, including both Aβ pathology and tau aggregation. Elevated levels of ceramides directly increase Aβ levels acting on β-secretase, a key enzyme in the proteolytic cleavage of Aβ precursor protein (APP). In turn, soluble and fibrillar forms of Aβ activate sphingomyelinases, enzymes that catalyze the breakdown of sphingomyelin to ceramides, and lead to further increase in Aβ generation. Ceramides are also linked to tau phosphorylation, in particular by modulating activity of protein phosphatase 2A, the major tau phosphatase in the human brain. Hence, preservation of neuronal ceramide homeostasis is of major importance for normal brain functioning. This chapter summarizes recent findings and potential targets for novel therapeutic approaches in AD regarding described devastating Aβ-ceramides-tau cascade.

**Keywords:** Ceramides, Aβ, phospho-tau, Alzheimer's disease

## INTRODUCTION TO ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease, and the leading cause of dementia syndrome in elderly people. In spite of major research efforts, current medicinal approaches have limited efficacy in alleviating symptoms, and do not cure the disease. Without improved therapeutic interventions, it is estimated that the number of affected people worldwide will reach 100 million by 2050 (Hanger et al., 2009). Slow and progressive degeneration of neurons in vulnerable brain areas such as entorhinal cortex, hippocampal formation and association regions of neocortex, results in gradual decline of cognitive abilities, loss of memory, impairment of normal behavior, and personality changes (Morrison and Hof, 1997; Robins Wahlin and Byrne, 2011; Sona et al., 2013; Levenson et al., 2014).

Neuropathologically, AD is characterized by the formation of extracellular senile plaques made of aggregated forms of neurotoxic amyloid β-peptide (Aβ), and intracellular deposits of abnormally phosphorylated tau protein in the form of neurofibrillary tangles (Šimić et al., 1998; Blennow et al., 2006; Gouras et al., 2010).

In the specific membrane microdomains called lipid rafts, Aβ is derived from the transmembrane amyloid precursor protein (APP) through the amyloidogenic pathway. APP is sequentially cleaved first by β-secretase (BACE1) and then by γ-secretase to yield Aβ peptide, a 39- to 43-amino acid

fragment. $A\beta_{42}$ is particularly prone to aggregation and self-assembles into a heterogeneous mixture of oligomers and protofibrils, finally deposited as fibrils in senile plaques (Vetrivel et al., 2005; Blennow et al., 2006; Thinakaran and Koo, 2008).

In addition to age that is the most significant risk factor for AD, alterations in the brain lipid profile and deregulation of lipid homeostasis have been recognized as important mediators of the disease onset and progression (Simons et al., 1998; Ferrer, 2009; Walter and Van Echten-Deckert, 2013; Fabelo et al., 2014; Yadav and Tiwari, 2014; Díaz et al., 2015). Epidemiological and animal studies suggest that a high-fat diet rich in cholesterol and saturated fats may disrupt central lipid metabolism, impair hippocampal morphology, and contribute to cognitive decline (Solfrizzi et al., 2005; Granholm et al., 2008; Morrison et al., 2010; Takechi et al., 2010; Stranahan et al., 2011; Morris and Tangney, 2014). Besides, the single most important genetic risk factor for the late-onset sporadic AD is a presence of the ε4 allele of the apolipoprotein E (apoE), a cholesterol transporter in the brain (Bertram et al., 2010; Liu et al., 2013a). Accordingly, cholesterol-lowering drugs like statins may prevent accumulation of Aβ *in vitro* and *in vivo*, and reduce the frequency of AD (Fassbender et al., 2001; Shepardson et al., 2011).

## Aβ AND ITS ROLE IN THE PATHOGENESIS OF AD

According to Aβ cascade hypothesis, formation of amyloid plaques in the AD brain has been long considered as the primary event in the pathogenesis of AD. However, as elderly person may deposit Aβ aggregates without signs of cognitive impairment, a relatively novel hypothesis emerged regarding the pathogenesis of cognitive decline. The new concept is relied on the small diffusible Aβ oligomers whose accumulation correlates much better with the disease onset and severity in comparison with the insoluble amyloid plaques (Aizenstein et al., 2008; Lesné et al., 2008; Rushworth and Hooper, 2011). Several neuronal receptors that bind Aβ oligomers have been identified, but mechanisms of Aβ neurotoxicity at the cellular level are poorly understood. Although evidence is accumulating that in normal physiological concentrations they positively regulate learning and memory, high levels of Aβ oligomers inhibit synaptic function, induce synaptic degeneration and dendritic spine loss, and alter hippocampal synaptic plasticity together with deficits in learning and memory (Lublin and Gandy, 2010; Larson and Lesné, 2012). In addition, they trigger the downstream pathways involved in

phospho-tau pathology (Hefti et al., 2013). Thus, isolated Aβ dimmers from the cortex of AD subjects may induce tau hyperphosphorylation at AD-relevant epitopes in primary hippocampal neurons, disrupt microtubule organization and provoke cytoskeleton collapse and neuritic dystrophy, all in the absence of amyloid fibrils (Jin et al., 2011). Likewise, soluble Aβ oligomers can facilitate development of tau pathology in animal models, such as transgenic mice model that expresses low levels of APP with the Arctic familial AD mutation (Chabrier et al., 2012). In these mice reduction of β-secretase decreases level of soluble Aβ oligomers, rescues cognition, and prevents tau accumulation and phosphorylation (Chabrier et al., 2012). Treatment with modulators of γ-secretase also can reverse Aβ-induced tau pathology and reduce phospho-tau levels in animal models of AD, including 3×Tg-AD mice, a model that develops Aβ pathology before tau pathology and tangle formation, as well as in transgenic mice expressing human amyloid precursor protein (hAPP) with Swedish and London mutations ($APP_{SL}$ mice) (McKee et al., 2008; Lanzillotta et al., 2011).

## PHOSPHO-TAU AND ITS ROLE IN THE PATHOGENESIS OF AD

Tau belongs to the family of microtubule-associated proteins. It has an essential role in promoting assembly and stability of axonal microtubules, thus influencing axonal transport, neuritic outgrowth, and neuronal shape (Esmaely-Azad et al., 1994; Choi et al., 2009). Although tau is encoded by a single gene, due to alternative mRNA splicing, several tau isoforms differing in their affinities for microtubules are expressed in human brain in a developmental, cell-specific and pathology-related manner (Takuma et al., 2003; Bullmann et al., 2009; Šimić et al., 2009). Besides affecting microtubule assembly by different isoforms, the biological activity of tau is modulated by its degree of phosphorylation. The longest tau isoform in human brain can potentially be phosphorylated at 80 serine and threonine residues and 5 tyrosine residues, that together comprise approximately 20% of the protein (Johnson and Stoothoff, 2004; Götz et al., 2010). Normal tau contains 2-3 moles of phosphate per mole of the protein for optimal activity in physiological conditions. In AD brain the phosphorylation stoichiometry is three to four times higher, that impairs tau ability to bind to microtubules and stimulate assembly of microtubule network. Enhanced phosphorylation not

only ends in a loss of tau function, but also results in a gain of toxic function, since aberrantly phosphorylated tau self-assemble into tangles of paired helical filaments and sequesters normal tau, and two other neuronal microtubule-associated proteins, MAP1A/MAP1B and MAP2, that slowly and progressively deregulates axonal transport, negatively affects axonal functions and leads to retrograde neurodegeneration and loss of synapses (Šimić et al., 1998; Alonso et al., 2001; Iqbal et al., 2005; Ballatore et al., 2007; Iqbal et al., 2010). The specific pattern of tau phosphorylation at each phosphorable amino acid is related to the severity of neuronal cytopathology, and appears in a stereotypical fashion, with certain regions being affected before others (Kimura et al., 1996; Augustinack et al., 2002; Braak et al., 2011).

Generally, the protein phosphorylation status depends on the balance between the activities of different protein kinases and protein phosphatases. In *in vitro* conditions, tau can be phosphorylated by numerous protein kinases; however, the number of kinases that actually phosphorylate tau is probably much lower *in vivo*. The total number of identified phosphorylated residues in aggregates from AD brain is at least 45 (Johnson and Stoothoff, 2004; Hanger et al., 2009). Tau is phosphorylated at various threonine and serine residues by proline-directed and non-proline directed kinases. Among them, as the most implicated in the abnormal hyperphosphorylation of tau, are glycogen synthase kinase-3β (GSK-3β), cyclin-dependent kinase 5 (cdk5), mitogen activated protein kinases (MAPKs), such as extracellular signal regulated kinase-1/2 (ERK1/2), JNK and p38, cyclic AMP-dependent protein kinase, calcium and calmodulin-dependent protein kinase-II, non-receptor tyrosine kinases and casein kinase 1δ (Maccioni et al., 2001; Li et al., 2006; Avila, 2006; Martin et al., 2013; Beharry et al., 2014). GSK-3β, cdk5 and ERK1/2, the three major proline-directed protein kinases, that catalyze phosphorylation of serine and threonine residues followed by proline, phosphorylate tau at a large number of the same sites (Iqbal et al., 2010). The considerable overlap of the phosphorylated residues suggests that the overall sum of multiple phosphorylation events is more important than the phosphorylation of each particular residue (Hanger et al., 2009; Mondragón-Rodríguez et al., 2012). It should be emphasized that ubiquitous, constitutively active kinase GSK-3, has two highly homologous isoforms, GSK-3α and GSK-3β, whose altered activity contributes to both key histopathological hallmarks of AD (Hooper et al., 2008; Lei et al., 2011). It is suggested that GSK-3α modulates production of Aβ by interfering with APP processing at the γ-secretase step, while GSK-3β co-localizes with neurofibrillary tangles in post-mortem AD brain, and is

considered the major tau kinase (Wagner et al., 1996; Yamaguchi et al., 1996; Phiel 2003; Jazvinšćak Jembrek et al., 2013).

Diverse phosphatases dephosphorylate tau by removing phosphate groups. Therefore, in addition to increased activity of protein kinases, downregulation of phosphatases in the AD brain also might lead to the hyperphosphorylation of tau and prevalence of abnormal, stable form prone to aggregation (Chohan et al., 2006). Activity and/or expression of protein phosphatases-1, -2A, -2B, and -5 (PP1, PP2A, PP2B, PP5), and phosphatase and tensin homolog deleted on chromosome 10 (PTEN), are altered in the AD brains. In particular, the overall phosphatase activity is reduced by half, and the most pronounced decrease is observed for PP2A, PP1 and PP5, 50%, 20% and 20%, respectively (Martin et al., 2013).

Phosphoprotein phosphatase 2A is considered the principal phospho-tau phosphatase in the human brain (Martin et al., 2009; Voronkov et al., 2011; Martin et al., 2013). It is a heterotrimeric protein that consists of a scaffolding A subunit, a regulatory B subunit, and a catalytic C subunit. Diversity of all these subunits leads to formation of more than 200 PP2A heterocomplexes. Activity of PP2A is regulated by phosphorylation, methylation and the binding of endogenous inhibitors. In AD, abnormalities of PP2A activity have been reported, including decreased protein levels of particular scaffolding and regulatory subunits, reduced methylation of catalytic subunits, up-regulation of PP2A inhibitors and loss of PP2A activity (Torrent and Ferrer 2012; Martin et al., 2013). Deposition of Aβ plaques is also subject to PP2A regulation. Namely, increased APP phosphorylation through decreased activity of PP2A towards phospho-APP may result in enhanced Aβ generation and amyloid plaque formation (Voronkov et al., 2011).

Key enzymes involved in the regulation of tau phosphorylation, GSK-3β and PP2A, have attracted much attention as promising targets for therapeutic intervention in AD (Martin et al., 2009; Llorens-Martín et al., 2014). Studies on transgenic mice have revealed that inhibition of GSK-3β may reduce hallmarks of tau pathology, including the level of tau phosphorylation, formation of aggregates, axonal degeneration and memory deficits (Noble et al., 2005; Onishi et al., 2011; Leroy et al., 2010; Noh et al., 2013). Besides, exposure of cortical neurons to GSK-3β inhibitors, not only reduced expression of tau protein and tau phosphorylation, but also provided neuroprotection against Aβ (Martin et al., 2009). Accordingly, brain permeable small molecule GSK-3 inhibitors progressed into clinical trials (Kramer et al., 2012; Georgievska et al., 2013). However, despite considerable efforts to develop therapeutic kinases inhibitors, success has been limited.

Besides other reasons, the structural homology of the ATP-binding pocket in GSK-3α and GSK-3β complicates the development of isoform selective inhibitors. Hence, efforts are redirected to an alternative approach aimed on the enhancement of the activity of specific tau phosphatases (Voronkov et al., 2011; Martin et al., 2013). A rational therapeutic strategy to increase PP2A activity is to inhibit its methylation (Sontag et al., 2004; Sontag et al., 2013). On the contrary, inhibition of PP2A activity by okadaic acid may reverse effect of GSK-3β inhibitors on tau decrease indicating that GSK-3β regulates extent of tau phosphorylation and total tau through PP2A (Martin et al., 2009). Furthermore, over-expression of inhibitor-2 of PP2A (I2PP2) causes abnormal hyperphosphorylation of tau that can be inhibited by memantine, a drug initially used against AD as a noncompetitive inhibitor of glutamate gated N-methyl-D-aspartate (NMDA) receptor channels (Chohan et al., 2006). It turns out that NMDA receptors may form complex with PP2A, and stimulation of NMDA receptors leads to dissociation of PP2A from the complex and reduces PP2A activity (Chan and Sucher 2001). Besides, in organotypic culture of rat hippocampal slices, memantine reduced abnormal hyperphosphorylation by inhibiting and reversing okadaic acid-induced PP2A inhibition (Li et al., 2004). Moreover, memantine and MK801, another NMDA antagonist, were effective against okadaic acid-induced neurotoxicity in rats, and prevented okadaic acid-induced changes in the expression of PP2A, tau, and GSK-3β (Kamat et al., 2013).

## INTERPLAY BETWEEN Aβ AND PHOSPHORYLATED TAU IN AD

In general, tau hyperphosphorylation is thought to be a later event in the course of AD. Several reports support the hypothesis that tau pathology results from increased Aβ accumulation, and in a certain way, GSK-3β represents a link between Aβ and tau pathologies (Terwel et al., 2008; Huang and Jiang 2009). Ma and co-workers (2006) have demonstrated that passive intracerebroventricular immunization with anti-Aβ antibody into the AD Tg2576 transgenic mouse model reduced soluble Aβ oligomers in correlation with reduced tau phosphorylation by GSK-3β. Antibody against amyloid oligomers also reduced activation of GSK-3β and protected neuronal SH-SY5Y cells against Aβ-induced neurotoxicity. In a similar study, it has been shown that soluble Aβ oligomers stimulated tau phosphorylation in mature

cultures of hippocampal neurons and in the neuroblastoma cells at epitopes that are characteristically hyperphosphorylated in AD (De Felice et al., 2008). They also confirmed that antibody against soluble Aβ oligomers prevents tau hyperphosphorylation. Furthermore, they induced tau hyperphosphorylation by a soluble aqueous extract containing Aβ oligomers from AD brains. Likewise, Resende and co-workers (2008) showed that soluble $A\beta_{42}$ oligomers, the most neurotoxic species, trigger tau phosphorylation and compromise cell survival by a mechanism that involves activation of GSK-3β. They demonstrated that $A\beta_{42}$ oligomers induce endoplasmic reticulum stress, and that $Ca^{2+}$ release from endoplasmic reticulum stores is further involved in GSK-3β activation and tau phosphorylation. In addition, inhibition of GSK-3β by natural extracts decreased Aβ-induced neurotoxicity and tau phosphorylation in cultured cortical neurons (Zhang et al., 2011). Vice versa, it seems that tau plays an important role in fibrillar Aβ-induced neuritic degeneration in central neurons, as tau-depleted neurons show no signs of degeneration in the presence of Aβ (Rapoport et al., 2002). Presenilin 1, a part of γ-secretase complex, may regulate phosphorylation of tau as it brings tau and GSK-3β in close proximity. Mutations in presenilin 1 that cause AD have increased ability to bind GSK-3β, and increase its tau-directed kinase activity (Takashima et al., 1998).

GSK-3β is downstream target of phosphatidylinositol 3-kinase (PI3-K)/Akt pathway. Kinase Akt phosphorylates GSK-3β at serine 9 and induces its inactivation in physiological conditions (Lei et al., 2011). In one study, injection of wortmannin, a specific PI3-K inhibitor, into the left ventricle of rat brains lead to overactivation of GSK-3 that correlated with hyperphosphorylation of tau, appearance of paired helical filaments in the hippocampus and impairment of spatial memory. Inhibition of GSK-3 activity by lithium chloride abolished these effects induced by PI3-K inhibition (Liu et al., 2003). Accumulation of $A\beta_{42}$ peptide in primary neuronal cultures results in the sequential decrease in the levels of phospho-Akt and inhibition of Akt pathway, downstream activation of GSK-3β and apoptosis (Magrané et al., 2005). In the same study down-regulation of Akt correlated with intracellular accumulation of Aβ in the Tg2576 AD mouse model, while overexpression of constitutively active Akt reversed the toxic effect of Aβ. Moreover, exposure to brain-derived neurotrophic factor (BDNF), an extracellular neurotrophic factor that is down-regulated in AD brains, induced a rapid tau dephosphorylation through a PI3-K/Akt and downstream GSK-3β signaling

mechanism. In cultured cortical neurons, Aβ at sublethal concentrations interferes with BDNF-induced PI3-K/Akt pathway that could be of importance for the future therapeutic approaches (Tong et al., 2004; Elliott et al., 2005). Interestingly, presenilin 1, by activating PI3-K/Akt signaling, promotes GSK-3 inactivation, suppresses tau phosphorylation at residues overphosphorylated in AD and prevents apoptosis (Baki et al., 2004).

As mentioned earlier, inhibition of GSK-3β emerged as an interesting target for therapeutic interventions in AD. Lithium, a non-specific GSK-3 inhibitor, reduces level of tau phosphorylation, formation of insoluble tau aggregates, and axonal degeneration *in vivo* (Noble et al., 2005). Related to Aβ pathology, lithium administration resulted in contradictory outcomes, with reported reduced, increased and unchanged Aβ levels (Su et al., 2004; Caccamo et al., 2007; Feyt et al., 2005; Parr et al., 2012). However, treatment with specific GSK-3 inhibitors resulted in more promising effects, including reduced amyloid deposition, protection of neurons in the entorhinal cortex and CA1 hippocampal subfield, and prevention of memory deficits (Serenó et al., 2009; Kramer et al., 2012; Georgievska et al., 2013).

## CERAMIDES AND THEIR ROLE IN THE PATHOGENESIS OF AD

Ceramides represent a heterogeneous class of bioactive sphingolipids. They consist of a sphingoid base backbone, primarily sphingosine, sphinganine and 4-hydroxysphinganine in humans, attached via an amide bond to different fatty acids, most commonly to palmitic and stearic acid acyl chain. Ceramides are the major molecules of sphingomyelin metabolism and the central second messengers of sphingolipid signaling (Zheng et al., 2006; Hannun and Obeid 2008; Ben-David and Futerman 2010; Mencarelli and Martinez-Martinez 2013). In association with other sphingolipids and cholesterol in lipid rafts, besides important structural role, they have essential function in transduction pathways that determine cell proliferation and differentiation, growth arrest, senescence and apoptosis. Furthermore, ceramides from lipid rafts may modulate synaptic transmission through the activation of postsynaptic protein phosphatases, affect exocytosis and trafficking of synaptic vesicles, and contribute to the maturation and maintenance of dendritic spines, thus acting as modifiers and regulators of

neuronal transmission (Yang 2000; Rohrbough et al., 2004; Carrasco et al., 2012).

Ceramides, together with other sphingolipids, comprise only a minority of membrane lipids, but even slight disturbances in their homeostasis may contribute to the development of neuropathological changes characteristic for AD, including production of Aβ, tau-associated alterations and neurodegeneration (Haughey et al., 2010; Mielke and Haughey 2012). At higher concentrations they act as effector molecules that activate the apoptotic cascade and promote cell death (Woodcock 2006; Portt et al., 2011; Yabu et al., 2015). Increase in ceramide levels is found in brains of AD patients in comparison to healthy age-matched control subjects (He et al., 2010; Fillipov et al., 2012), as well as in the cerebrospinal fluid of patients with AD (Satoi et al., 2005). Moreover, enhanced levels of ceramides are found in patients with mild to moderate symptoms, indicating that alterations of sphingolipid profile occur early in the course of AD (Han et al., 2002; Cutler et al., 2004).

Ceramides may be formed via three different pathways: degradation, *de novo* synthesis and recycling. In the catabolic pathway, ceramides are generated through the sphingomyelin hydrolysis by sphingomyelinases, a family of sphingomyelin-specific phospholipase C enzymes that differ in subcellular localization, optimal pH and cation dependence (Marchesini and Hannun 2004; Jana et al., 2009; Mencarelli and Martinez-Martinez 2013). Neutral sphingomyelinase, the key enzyme with respect to AD, is membrane-bound, $Mg^{2+}$-dependent protein, activated by a variety of stress conditions that probably mediates effects of oxidative stress and inflammatory cytokines on ceramide content and neuronal survival in AD (Jana et al., 2009; Barth et al., 2012; Yabu et al., 2015). In *de novo* pathway, ceramide synthesis begins with the condensation of serine and palmitoyl-CoA, the rate-limiting reaction of the pathway, by a serine palmitoyltransferase (Young et al., 2012; Mencarelli and Martinez-Martinez 2013). Finally, ceramides may be generated in an alternative pathway that relies on the ceramide regeneration from complex sphingolipids such as glycosphingolipids. Sphingosine, the end product of sphingolipid catabolism, is mostly salvaged through the reacylation, resulting in the generation of ceramides. This pathway is also known as the salvage pathway as catabolic fragments are reused to yield ceramides (Kitatani et al., 2008; Novgorodov and Gutz 2011).

Permeable ceramide analogues, in particular short-chain C2- and C6-ceramide, have been widely used in studies aimed to elucidate molecular mechanisms of ceramide-mediated neurodegenerative changes in AD (Toman et al., 2002; Puglielli et al., 2003; Tang et al., 2011; Zhang et al., 2012;

Czubowicz and Strosznajder 2014). There are numerous downstream targets of ceramides. They act as regulators of ceramide-activated protein kinases (CAPK), ceramide-activated protein phosphatases (CAPP), mitogen activated protein kinases (MAPKs), protein kinase C-zeta, cathepsin D, and diverse phospholipases (Mencarelli and Martinez-Martinez 2013; Czubowicz and Strosznajder 2014). Ceramide-activated protein phosphatases, including PP1 and PP2A, are alosterically activated by ceramides and exert important roles in regulation of ceramide-induced apoptosis (Chalfant et al., 1999; Chalfant et al., 2004). Ceramides may modulate cell death through a variety of signaling pathways. For example, they decrease antiapoptotic signaling by inhibiting activity of Akt kinase (Zhou et al., 1998). In primary cortical neuronal cells ceramide-induced signal transduction changes involve Akt dephosphorylation and inactivation, followed by dephosphorylation of proapoptotic regulators and GSK-3β, mitochondrial depolarization and permeabilization, release of cytochrome c, and caspase-3 activation (Stoica et al., 2003). Ceramide-induced activation of GSK-3β via dephosphorylation at serine 9 also leads to caspase-2 activation and the subsequent death cascade, that can be abolished by GSK-3β inhibitors and GSK-3β knockdown. It turns out that ceramide-induced PP2A indirectly dephosphorylates and activates GSK-3β by inhibiting pro-survival PI3-K/Akt pathway (Lin et al., 2007). C2-ceramide-induced negative regulation of Akt pathway, due to enhanced dephosphorylation of Akt by ceramide-activated protein phosphatases PP2A, is also reported in neuronal growth factor (NGF)-treated PC12 cells (Salinas et al., 2000). Specific B regulatory subunit B55α targets the PP2A holoenzyme and selectively regulates Akt phosphorylation and cell survival (Kuo et al., 2008). Studies on human neuroblastoma cell line (SH-SY5Y cells) indicated that through inhibition of PI3-K/Akt pathway, ceramides not only activates GSK-3β and proapoptotic regulator Bad as downstream targets, but also decreases anti-apoptotic protein Bcl-2, increases pro-apoptotic protein Bax, and significantly enhances the level of free radicals consequently reducing the viability (Kim et al., 2007; Czubowicz and Strosznajder 2014). Effects of ceramides in neuronal apoptosis signaling are often related to Bax activation and its redistribution to mitochondria (Falluel-Morel et al., 2004; Jin et al., 2008). Furthermore, S-nitrosoglutathione, a potent endogenous antioxidant, may rescue primary cortical cultures against Aβ- and ceramide-induced toxicity through the activation of PI3-K/Akt pathway that results in the induction of Bcl-2 and antioxidant enzyme thioredoxin (Ju et al., 2005). In cells expressing functional Bcl-2, the mechanism of death action for ceramide may also involve, at least in part, inactivation of Bcl-2 via dephosphorylation

by mitochondrial PP2A (Ruvolo et al., 1999). However, in contrast to generally accepted beneficial role of Act on neuronal survival, up-regulation of Akt with concomitant hyperphosphorylation of specific Akt targets have been observed in AD brains in association with reduced and altered distribution of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a negative Akt regulator, indicating deregulation of Akt and PTEN signaling in AD (Griffin et al., 2005; Ismail et al., 2012).

In addition to Akt kinase, ceramides may exert their biological effects by modulating c-Jun N-terminal kinases (JNK)-pathways (Cui et al., 2007). JNK and transcription factor c-Jun are important regulators of cell death program in post-mitotic neurons. In differentiated PC12 cells, increased ceramide formation after activation of neutral sphingomyelinase was associated with JNK phosphorylation and neuronal apoptosis through caspase-3 activation (Soeda et al., 2004). In cerebellar granule cells, a JNK-inhibitor L-JNK1 was able to block C2-ceramide-induced stimulatory effect on caspases-9 and -3, and prevent increase in ceramide-evoked Bax expression and cell death (Falluel-Morel et al., 2004). C2-ceramide-induced JNK activation by phosphorylation results in c-Jun phosphorylation. Namely, phospho-JNK is localized in neurites, and translocates to the nucleus where phospho-c-Jun concurrently appears upon ceramide-activated apoptosis (Willaime-Morawek et al., 2003). Furthermore, it seems that p38 and JNK/c-Jun pathways cooperate in initiation of neuronal apoptosis. In primary cortical cultures, C2-ceramide rapidly decreases phosphorylation of extracellular signal regulated kinases (ERKs) and their upstream activators MAPK kinases (MEKs), and increases JNK and p38 phosphorylation before and during caspase-3-activation, together with upregulation of c-jun, c-fos, and p53 mRNA (Willaime et al., 2001; Willaime-Morawek et al., 2003). Accordingly, JNK-, p38-, and p53-inhibitors efficiently block C2-ceramide-induced apoptotic signaling and protect neuronal cells against death by attenuating mitochondrial release of cytochrome c and caspase-3 activation (Stoica et al., 2005; Czubowicz and Strosznajder 2014). In another study, neuroprotection mediated by antioxidants was achieved through ERK signaling (Ju et al., 2005).

## INTERPLAY BETWEEN Aβ AND CERAMIDES IN AD

Because ceramides act as pro-apoptotic molecules, sphingomyelinase up-regulation promotes loss of neuronal cells. As already emphasized, neutral

sphingomyelinase is considered as the key enzyme of sphingolipid metabolism, particularly sensitive to oxidative stress and proinflammatory cytokines, both of which are hallmarks of AD that can enhance neurodegenerative processes (Krönke 1999; Jana and Pahan, 2004). Activity of membrane-associated neutral sphingomyelinase increases with age, as demonstrated in hippocampus and striatum (Crivello et al., 2005). Elevated expression of acidic sphingomyelinase is also observed in the AD brain in comparison with age-matched normal subjects, leading to a depletion of sphingomyelin and increase of ceramides. Moreover, correlation is found between the brain acidic sphingomyelinase and the levels of Aβ and phosphorylated tau (He et al., 2010). Therefore, enhanced accumulation of ceramides during physiological aging and in the AD patients, due to disturbances in sphingolipid metabolism, can magnify toxic action of Aβ. Vice versa, treatment with Aβ oligomers can directly activate neutral sphingomyelinase, increase ceramide content, down-regulate sphingomyelin level and induce apoptosis (Grimm et al., 2005; He et al., 2010).

A large body of evidence supports the conclusion that Aβ triggers apoptotic cascade via the sphingomyelin/ceramide pathway. Ju and co-authors (2005) established the Aβ→ neutral sphingomyelinase activation → ceramide increase → generation of free radicals → apoptosis sequence in primary cultures of fetal rat cortical neurons, and showed that exogenous application of antioxidants such as thioredoxin and S-nitrosoglutathione may exert remarkable neuroprotective efficacy against Aβ/ceramide-induced toxicity. Study of Jana and Pahan (2004) also underlined the importance of neutral sphingomyelinase, but not acidic sphingomyelinase, in Aβ-induced apoptosis and cell death of human primary neurons. They showed that $A\beta_{42}$ induced production of ceramides via activation of NADPH oxidase and production of superoxide radicals that further activated neutral sphingomyelinase. In addition, the critical role of a redox-sensitive cytosolic calcium-dependent phospholipase A2 (cPLA2)-arachidonic acid (AA) pathway in Aβ-induced cell death has also been demonstrated in rat cortical neurons (Malaplate-Armand et al., 2006). Namely, polyunsaturated fatty acids like arachidonic acid are involved in the regulation of sphingomyelinase activity. This study supported the role of both neutral and acidic sphingomyelinases in Aβ-induced apoptosis, as demonstrated by the direct measurement of their enzymatic activities, by specific neutral and acidic sphingomyelinase inhibitors, and by gene knockdown using antisense oligonucleotides.

On the other hand, Aβ production is determined by the endogenous ceramide content. In particular, it is demonstrated that C6-ceramide increases synthesis of Aβ. Similarly, enhancement of endogenous ceramide content by sphingomyelinase treatment promoted synthesis of Aβ. It turns out that ceramides promote secretion of Aβ by affecting β cleavage of APP through post-translational stabilization of β-secretase (Puglielli et al., 2003). Accumulation of sphingolipids also may stimulate γ-secretase activity (Tamboli et al., 2011a). In addition, increase in sphingolipids decreases the capacity of cells to clear potentially amyloidogenic fragments of APP during autophagy and thus could promote Aβ accumulation in endosomal and lysosomal compartments (Tamboli et al., 2011b). Conversely, pharmacological depletion of ceramides and complex sphingolipids can markedly reduce secretion of endogenous APP and Aβ in different cells lines, including neuroblastoma SH-SY5Y cells, by affecting maturation and cell surface transport of APP, and its proteolytic processing (Puglielli et al., 2003; Tamboli et al., 2005). *In vivo*, sphingomyelinase activity and ceramide levels are found increased in hippocampus and neocortex 7 days after single Aβ administration (Allesenko et al., 2004). Thus, ceramide-induced increase in Aβ production leads to sphingomyelinase activation, pointing to the presence of vicious cycle in which initial ceramide increase leads to more pronounced ceramide accumulation via Aβ.

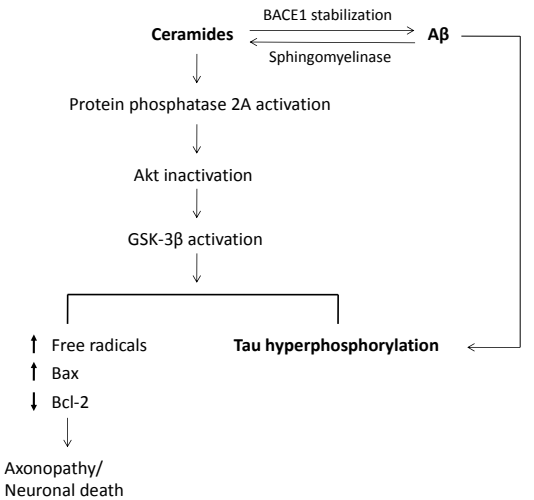


Figure 1. Interplay between ceramides, Aβ and hyperphosphorylated tau in AD. (See the text for details).

Recent studies have revealed interesting crosstalk between neurons and glia in sphingolipid metabolism and Aβ accumulation. In general, deregulation of ceramide metabolism in astrocytes induces Aβ secretion and tau phosphorylation in neurons. Firstly, Patil and co-authors (2006) have demonstrated that conditioned medium from palmitic acid-treated astrocytes upregulated β-secretase (BACE1) and consequently increased APP processing in primary rat cortical neurons, although in neurons exposed directly to palmitic acid there was no change in BACE1 activity. Based on the finding that upregulation of BACE1 can be abolished in the presence if antioxidants, it is concluded that astroglia-mediated oxidative stress is responsible for increased amyloidogenesis. Later, it is found that palmitic acid promotes *de novo* synthesis of ceramides in astroglia (Patil et al., 2007). Finally, it is demonstrated that generated ceramides induce production of pro-inflammatory cytokines in astrocytes. These soluble mediators after release into the surrounding medium activate neuronal sphingomyelinase, upregulate ceramide generation, BACE1 activity and Aβ generation (Liu et al., 2013b).

Sphingomyelin/ceramide pathway is considered as a promising approach in AD therapy. Inhibition of ceramide production may reduce Aβ generation and neurotoxicity. Thus, inhibition of astroglial ceramide synthesis by L-cycloserine, a serine palmitoyltransferase inhibitor that prevents *de novo* ceramide synthesis, blocked the aforementioned palmitic acid-induced up-regulation of BACE1, production of Aβ and hyperphosphorylation of tau (Patil et al., 2007). Similarly, in TgCRND8 mice, an early-onset AD model, the subcutaneous administration of L-cycloserine downregulated cortical $A\beta_{42}$ and hyperphosphorylated tau levels, in parallel with reduction of ceramide content (Geekiyanage et al., 2013).

As increase in ceramides leads to increase in Aβ, and Aβ affects tau phosphorylation, in vicious cycle enrichment in ceramide content would also promote tau pathology (Figure 1). Accordingly, upregulation of sphingo-myelinases correlates with increased tau hyperphosphorylation in the AD brain (He et al., 2010). Moreover, ceramides may affect tau hyperphosphorylation by modulating PP2A activity. It has been shown that ceramides activate PP2A in rat T9 glioma cells and rat brain (Dobrowsky et al., 1993). Inhibitor 2 of PP2A (I2PP2A), via interactions with ceramides, exerts decreased association with PP2A, and prevents inhibition of PP2A activity (Mukhopadhyay et al., 2009). Modulation of PP2A activity is considered as a promising next generation focus in AD therapy as it can be safely, selectively and effectively targeted by pharmaceutical interventions. As reviewed by Voronkov and coauthors (2011), various compounds may enhance PP2A activity through

different mechanisms, such as direct allosteric activation, inhibition of binding of PP2A inhibitors and via post-translational modifications. It seems that lithium also exerts its effects via inhibition of PP2A activity, in particularly by down-regulating ceramide-induced PP2A methylation (Chen et al., 2006).

## CONCLUSION

Ceramides, the major molecules of sphingolipid metabolism, act as lipid second messengers, affecting various aspects of neuronal physiology such as cell differentiation, growth, survival, senescence and apoptosis. Alterations of brain lipid profile and disturbed ceramide metabolism are hallmarks of the AD brain, strongly indicating importance of ceramide homeostasis for normal brain functioning. Increase in ceramide content is already present in patients with mild and moderate symptoms of AD. Thus, it seems that alterations in ceramide metabolism occur early in the course of AD, and implies an important role of enhanced ceramide level in the AD onset and progression. In vicious interrelated networks of biochemical events, ceramides are inseparably associated with the key histopathological aspects of AD, Aβ accumulation and tau hyperphosphorylation, which ends in neurodegeneration. Of particular importance for Aβ-related neurotoxicity is the effect of ceramides on β-secretase stabilization, that reinforces APP processing and Aβ deposition. As soluble and fibrillar forms of Aβ can activate neuronal sphingomyelinase, initial ceramide enhancement promotes further ceramide accumulation. On the other hand, ceramides may promote tau hyperphosphorylation via PP2A activation, Akt inactivation and subsequent GSK-3β activation, the key tau kinase.

In spite of the major research efforts, there is still no effective AD treatment. Nowadays it is generally accepted that successful development of novel therapeutic interventions must rely on the inhibition of several molecular targets. Strategies aimed to reduce ceramide levels have the potential to prevent Aβ burden and slow down progression of the disease, in combination with approaches aimed to inhibit activity of GSK-3β and enhance the activity of PP2A.

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