INVESTIGATING THE EFFECTS OF CERAMIDE AND SPHINGOMYELINASE ON THE NMDA RECEPTOR OF HIPPOCAMPAL PYRAMIDAL CELL

by

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ABSTRACT

INVESTIGATING THE EFFECTS OF CERAMIDE AND SPHINGOMYELINASE ON THE NMDA RECEPTOR OF HIPPOCAMPAL PYRAMIDAL CELL

Ceramide is a structural membrane component which plays important roles in carrying the message about the fate of cell such as apoptosis, differentiation or cell growth. Also, hippocampus is a very important region for transferring the information in central nervous system and NMDA receptors are related with learning and memory. Various pathological conditions occur as a result of differences in neuronal activities in hippocampus. Therefore, in this study, the effects of C2-ceramide (10 μM), C2-dihydroceramide (10 μM) and sphingomyelinase (30 mU/ml, 50 mU/ml) on NMDA receptors of hippocampal CA1 pyramidal neurons were investigated by using the patch-clamp technique. It was observed that C2-ceramide had a depression effect on NMDA currents. C2-dihydroceramide, which is the inactive form of C2-ceramide, depressed the amplitude of NMDA currents, as well. In addition to that, sphingomyelinase which activates the formation of ceramide applied at two different concentrations. Both of them decreased the amplitude of NMDA current, so they had the same effect as C2-ceramide. Besides being a second messenger in the cell, C2-ceramide and also C2-dihydroceramide may have some modulatory effects on the cell membrane. Therefore, it is possible that sphingomyelinase interacts with the cell membrane directly to form ceramides. In conclusion, ceramide molecule plays an important role in the regulation of glutamate mediated NMDA currents which plays important roles in hippocampal plasticity, where clarification of its role requires further investigations.

Keywords: C2-ceramide, C2-dihydroceramide, sphingomyelinase, hippocampus, NMDA currents, patch-clamp technique.

ÖZET

SERAMİD VE SFİNGOMYELİNAZIN HİPPOKAMPAL PİRAMİDAL HÜCRELERİN NMDA RESEPTÖRLERİ ÜZERİNE ETKİSİNİN ARAŞTIRILMASI

Hücre zarının yapısal bir bileşeni olan seramid, apoptoz, hücre farklılaşması veya hücre büyümesi gibi hücrenin geleceği ile ilgili mesaj taşınmasında önemli bir rol oynar. Ayrıca, hippokampus, merkezi sinir sisteminde bilgi akışını sağlayan çok önemli bir bölgedir ve NMDA reseptörleri öğrenme ve hafıza yetileri ile bağlantılıdır. Hippokampustaki nöron aktivilerindeki değişikler farklı patolojik durumlara neden olur. Bundan dolayı, bu çalışmada C2-seramid (10 μM), C2-dihidroseramid (10 μM) ve sfingomyelinaz (30 mU/ml, 50 mU/ml) uygulamalarının hippokampal CA1 piramid hücrelerindeki NMDA reseptörleri üzerine etkisi patch-clamp tekniği kullanılarak araştırıldı. C2-seramidin NMDA akımları üzerine baskılayıcı bir etkisi olduğu gözlemlendi. Öte yandan, C2-seramidin inaktif formu olan C2-dihidroseramid de NMDA akımının genliğini baskıladı. Buna ek olarak, seramid molekülünün oluşumunu aktive eden sfingomyelinaz enzimi iki farklı konsantrasyon olarak uygulandı. Her iki uygulama da NMDA akımının genliğini düşürdü, dolayısıyla iki uygulama da C2-seramid ile aynı etkiyi yarattı. Hücrede ikincil mesaj taşıyıcısı olmanın yanı sıra, C2-seramid ve hatta C2-dihidroseramid hücre zarında etkin özelliklere sahip olabilir. Dolayısıyla sfingomelinazın seramid oluşumu için doğrudan hücre zarıyla etkileştiği düşünülebilir. Sonuç olarak, seramid molekülü hippocampal plastisitede önemli rol oynayan glutamat kontrolündeki NMDA akımının düzenlenmesinde önemli bir role sahiptir ve bu konuda ileri araştırmalara gereksinim vardır.

Anahtar Sözcükler: C2-seramid, C2-dihidroseramid, sfingomyelinaz, hipokampus, NMDA akımları, patch-clamp tekniği.

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DG	Dentate Gyrus
CA	Cornu Ammonis
MFs	Mossy Fibers
Glu	Glutamate
CNS	Central Nervous System
iGluRs	Ionotropic Glutamate Receptors
NMDA	N-methyl-D-aspartate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
Kainate	2-carboxy-3-carboxymethyl-4-isopenylpyrrolidine
AP5	2-amino-5-phosphonopentanoic acid
CPP	3-|($\pm$-2carboxypiperazin-4-y1| propyl-1-phosphonic acid)
LTP	Long Term Potentiation
GPCR	G-Protein Coupled Receptor
ATD	Amino-Terminal Domain
SM	Sphingomyelin
GlcCer	Glucosylceramide
Gal	Galactose
GlcNAc	N-acetylglucosamine
GalNAc	N-acetylgalactosamine
Fuc	Fucose
Neu	N-acetylneuraminic acid
Cer	Ceramide
Sph	Sphingosine
S1P	Sphingosine 1-phosphate
SMase	Sphingomyelinase
CDase	Ceramidase
CAPK	Ceramide-Activated Protein Kinase
CAPP1	Ceramide-Activated Protein Phosphatase 1

CAPP2A	Ceramide-Activated Protein Phosphatase 2A
N-SMase	Neutral Sphingomyelinase
A-SMase	Acid Sphingomyelinase
TNFα	Tumor Necrosis Factor-α
NGF	Nerve Growth Factor
JNK	Jun Kinase
SPT	Serine Palmitoyltransferase
CERT	Ceramide Transfer Protein
ACSF	Artificial Cerebrospinal Fluid
ICF	Intracellular Fluid
DNQX	7-Dinitroquinoxaline-2,3-dione
DMSO	Dimethylsulfoxide
PBS	Phosphate Buffered Saline
C2-Cer	C2-ceramide
C2-DHCer	C2-dihydroceramide
PCP	Phencyclidine
EPSCs	Excitatory Post-Synaptic Currents
PS	Population Spike
fEPSP	field Excitatory Post-Synaptic Potential
AP	Action Potential
LTD	Long Term Depression
SC	Stratum Corneum
IL	Interleukin
TGF	Transforming Growth Factor
TEWL	Transepidermal Water Loss
GCS	Glucosylceramide synthase
SK	Sphingosine Kinase

1.   INTRODUCTION

1.1   Hippocampal Formation

The cerebral cortex is a well developed structure of the human brain and it is effective in complex brain functions; like, consciousness, learning-memory, and language [1]. It is a part of the grey matter and composed of unmyelinated nerve cells. Beside this, the neocortex is the outer region of the cerebral cortex. It includes the frontal, parietal, occipital and temporal lobes [2].

The hippocampus (greek *hippocampus:seahorse*) is an incurved seahorse shaped structure of cerebral cortex [3]. There are two hippocampi, located on each side of the brain so it is bilateral. This structure is a part of the limbic (latin *limbus: border*)system and it exists beneath the neocortex, on the basal medial surface of the temporal lobes. It continuous from the amygdala to the septum, along the temporal lobes [4]. The axis from the amygdala to the septum defines the septotemporal axis of the hippocampus. The hippocampus and subiculum which is in the inferior part of the hippocampus, are indicated as the hippocampal formation. This structure is phylogenetically one of the oldest structures of the brain. In rat and other lower mammals, the hippocampus is a smooth structure, on the other hand in primates and other higher mammals, it has deeply folded and wrinkled structure [5].

The hippocampus has a unique morphology and consists of two regions; namely, the dentate gyrus (DG) and the *Cornu Ammonis* (CA). These regions are anatomically different which have distinct morphology, cell size and shape, also their connectivity and electrophysiological properties. The inner part of the DG is called *hilus* or hilar region. The CA region is composed of four subfields: CA1, CA2, CA3 and CA4 [6].

There is a main cell layer in each of these regions which is known as principal cell layer. The nerve cells of the principal cell layer for the CA regions are the pyra-
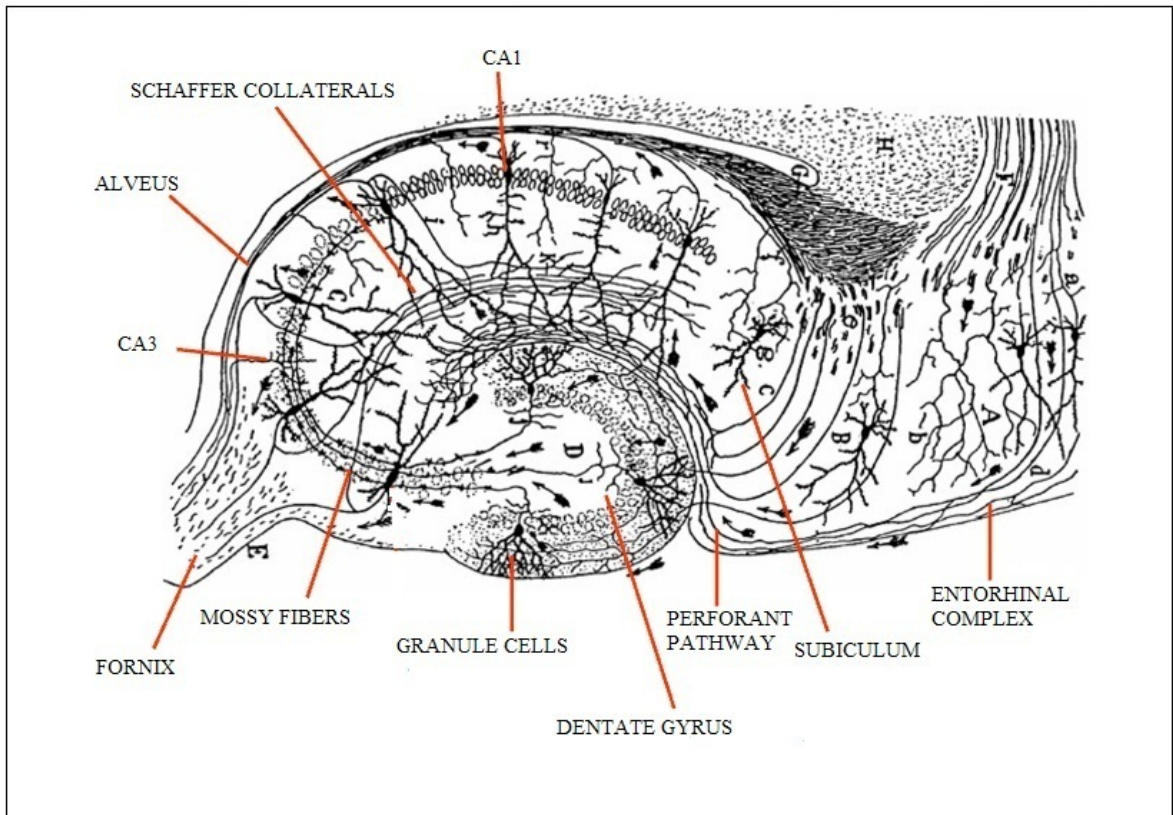
Figure 1.1 Hippocampal formation (Adapted from Cajal, 1911) [3].

midal cells and for the DG region; it is granule cells. These layers are called *stratum pyramidale* and *stratum granulosum*, respectively and they contain the cell bodies of the granule and pyramidal cells. Both of these two types of cells are excitatory and glutamatergic.

1.1.1   Hippocampal Subfields

1. *The Dentate Gyrus*

   The layers or *strata* of dentate gyrus (DG) are the polymorphic layer, the*stratum granulosum* and *stratum moleculare*. The *stratum granulosum* or the granule cell layer is the principal cell layer of DG and this layer contains the cell bodies of the granule cells. The *stratum moleculare* includes the proximal dendrites of the granule cells. The mossy fibers (MFs), the axons of the granule cells, project synaptic contacts with the pyramidal cells of the CA3 region [7].

2. *The Cornu Ammonis*

   The *strata* of the *Cornu Ammonis* (CA) are the *stratum moleculare, stratum lacunosum (lacunosum-moleculare), stratum radiatum, stratum lucidum, stratum pyramidale, stratum oriens* and the *alveus*. The *stratum pyramidale* or the pyramidal cell layer is the principal cell layer of CA region and this layer contains the cell bodies of the pyramidal cells. The CA region was divided into 4 subfields: CA1, CA2, CA3 and CA4 by Lorente de No (1934) [6]. The CA1 region is nearby the subiculum and the CA3 region is next to the fimbria/fornix and choroid plexus. The CA2 region is in between CA1 and CA3 regions, also it is small region. The CA4 is placed in the *hilus* of DG.

   The pyramidal cell bodies have triangular shape. While their basal dendrites are found in the *stratum oriens*, the apical dendrites of the pyramidal cells are located in the *stratum moleculare*.

1.2 Excitatory Pathways in Hippocampus

The major hippocampal projections are organized in both septotemporal and transverse axis of hippocampus. Furthermore, some hippocampal projections, the associational projections which are originated from dentate gyrus, are organized to unite different levels of hippocampal formation [8, 9]. The hippocampus receives its afferences from several neighboring brain areas, however the entorhinal cortex is the main afferent way of the hippocampus. The connection of the main afferent ways of the hippocampus with the hippocampal granule and pyramidal cells is "the lamellar tri-synaptic circuit".

1.2.1 Tri-Synaptic Circuit

The tri-synaptic circuit is organized along the septotemporal axis of the hippocampus [10]. The first connection of the tri-synaptic circuit is the main afference of the hippocampus in which the perforant fibers come along in the *stratum moleculare* of the DG and project to the granule cells. The second connection of the circuit is formed by MFs which are the axons of the granule cells. These axons make a link to the dendrites of the pyramidal cells of CA3. The third connection of the circuit is organized between the pyramidal cells of CA3 and CA1 via the collateral which is sent by CA3 pyramidal cells. This link is called Schaffer collateral and it goes through the *stratum radiatum*. Associated with this concept is the assumption that generally hippocampal information flows through the tri-synaptic circuit unidirectly. On the other hand, even though the initial entry point of information flow is the dentate gyrus, it is reported that CA3 region is the second entry point to receive information from afferents to hippocampus [11].

In addition to the tri-synaptic network, there is a network of projections that connects one region to one or two regions upstream [12]. The pyramidal neurons of the CA1 and CA3 regions are innervated by the perforant fibers and temporo-ammonic projections which are the axons of the pyramidal cells of the layer III of the entorhinal
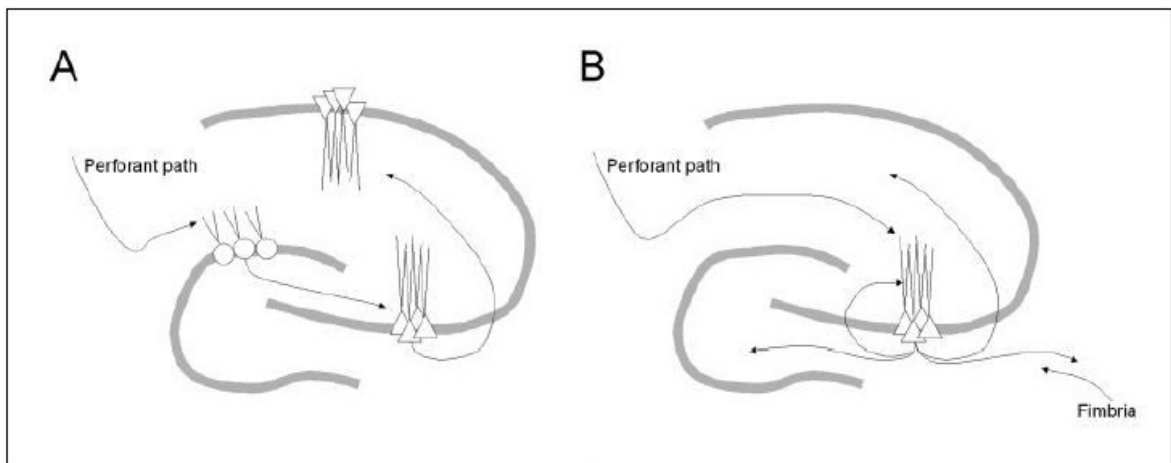
Figure 1.2 "Trisynapto-centric" and "CA3-centric" information pathways [11].

cortex. The CA3 pyramidal cells project to the subiculum and entorhinal cortex as well as it projects to the CA1. Also, the pyramidal cells of the CA3 connect to each other via axons, collateral fibers or recurrent fibers. Besides, the CA1 pyramidal cells connect to the subiculum and entorhinal cortex via fibers as the CA3 pyramidal cells do. However, there is an exception to this concept that the granule cells of the DG only innervate the CA3 pyramidal cells [13]. The main output of the hippocampus is formed by the the axons of the pyramidal cells run through the *alveus* to the fimbria/fornix.

1.2.2 Hippocampal Afferences

Besides the afferences from the entorhinal cortex, there are some other afferences of the hippocampus which are:

- the hypothalamus which projects to the DG and CA2 regions. This afferences connect the hippocampus via the fibria/fornix [5].

- the septal nucleus median and nucleus of the diagonal band of Broca which project to all hippocampal regions, especially the DG and CA3 regions [14]. These afferences, cholinergic and GABAergic, emphasize double GABAergic inhibition in the septo-hippocampal network.

- the contralateral hippocampus which is a network of afferent GABAergic fibers, the commissural fibers, and originating from the contralateral hippocampus. This afference uses the *corpus callosum* to enter other hemisphere, also it uses the fibria/fornix to enter the hippocampus. The commissural fibers project to the DG and are located through the *stratum moleculare* of the DG [5].

1.2.3 Hippocampal Interneurons

There are mostly symmetrical synaptic contacts between granule cells and the interneurons, mossy cells and basket cells in the DG [15]. Symmetrical contacts indicate a histological criteria of inhibitory synapses [5]. The perforant path, afferences from the entorhinal cortex, establish synaptic contacts with not only granule cells but also interneurons in the DG molecular layer. These interneurons run through the layer beneath the DG, the subgranular zone [16, 17]. Pyramidal cells in the CA regions are innervated by basket cells so symmetrical synapses are established [18]. The CA4 region which is located in the hilar region of the DG consists of CA3-like pyramidal neurons that do not receive inhibitory input from basket cells [5].

1.2.4 Feed-Back Inhibition and Feed-Forward Inhibition

In the CA regions, the basket cells innervate the pyramidal cells and they establish the symmetrical synapses which are innervated by commissural fibers and collateral fibers of pyramidal cells [5]. These commissural and collateral fibers of pyramidal cells form asymmetrical synapses with the interneurons and asymmetrical synapses are indicators of excitatory synapses [5]. Associated with this concept pyramidal cells control the activation of inhibitory neurons and the inhibition is qualified "feed-back inhibition".

The pyramidal cells of CA3 are innervated by the MFs and also inhibitory interneurons are innervated by the MFs fibers and their collaterals [19]. Asymmetrical

synaptic contact is formed by these MF endings at the level of dendritic tree of interneurons instead of the cell body level [19]. If the MFs activate the inhibitory interneurons in CA3 region, there is an inhibition of the pyramidal cells and this control mechanism is called "feed-forward inhibition" [20].

1.3   Glutamate Receptors in Hippocampus

Glutamate (Glu) is the most common neurotransmitter in the mammalian central nervous system (CNS) and it is acting on glutamate receptors. In nerve cells, Glu is synthesized from glutamine (Gln) by glutaminase. GluRs are divided into two major classes as ionotropic (ligand-gated cation channels) and metabotropic (G-protein coupled) receptors [21].

GLUTAMATE

Ionotropic glutamate receptors (iGluRs)			Metabotropic glutamate receptors (mGluRs)		
NMDA receptor	AMPA Receptor	Kainate Receptor	Group I	Group II	Group III
NR1	GluR1	GluR5	mGluR1	mGluR2	mGluR4
NR2A	GluR2	GluR6	mGluR5	mGluR3	mGluR6
NR2B	GluR3	GluR7			mGluR7
NR2C	GluR4	KA-1			mGluR8
NR2D		KA-2			
NR3A					
NR3B					
↓	↓	↓	G_q / G_{11}		G_i / G_o
Ca^{2+}	Na^+	Na^+	↓		↓
Na^+	(Ca^{2+})	(Ca^{2+})	↟PLC		↟AC
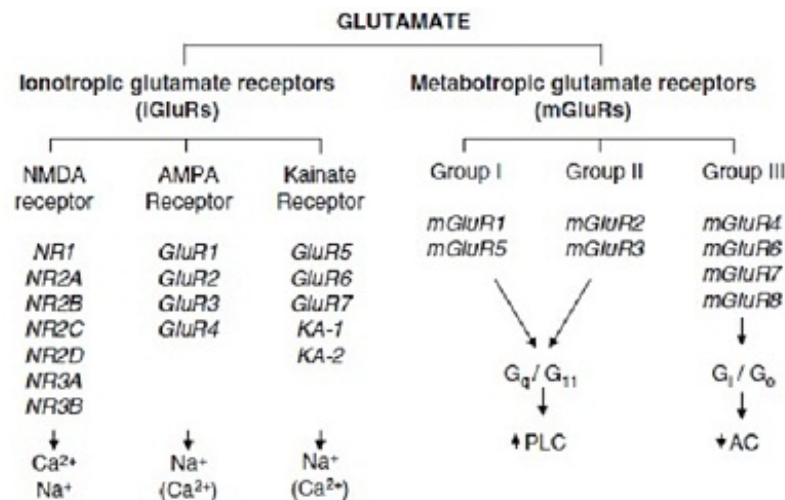
Figure 1.3 Overview of both the ionotropic and metabotropic glutamate receptors, their subunits. Cation-permeable ionotropic receptors and G-protein coupled metabotropic receptors are activated by glutamate [21].

1.3.1   Ionotropic Glutamate Receptors

Ionotropic receptors are coupled to ion channels or ligand gated ion channels. These type of receptors open rapidly so they form fast and large changes in the conductance of the membrane which leads to the depolarization and hyperpolarization of

the membrane. The interaction of fast-acting ionotropic glutamate receptors transmit the nerve activity. There are three major types of ionotropic glutamate receptors (iGluRs)(Fig.1.4) [22]. These iGluRs are named in function of their agonists which were identified to activate them selectively are called *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate (AMPA) and 2-carboxy-3-carboxymethyl-4-isopenylpyrrolidine (kainate) receptors. IGluRs are multimeric receptors which are composed of a combination of 4 to 5 subunits. They cooperate with sodium and calcium channels. Magnesium ion has an effect on the NMDA receptor as a voltage-dependent blocker. The activation of iGluRs mediates the opening of sodium and calcium ion channels. The depolarization of the membrane potential of the post-synaptic membrane is induced by the influx of sodium and calcium inside the cells so this triggers the transmission of nerve activity [5].
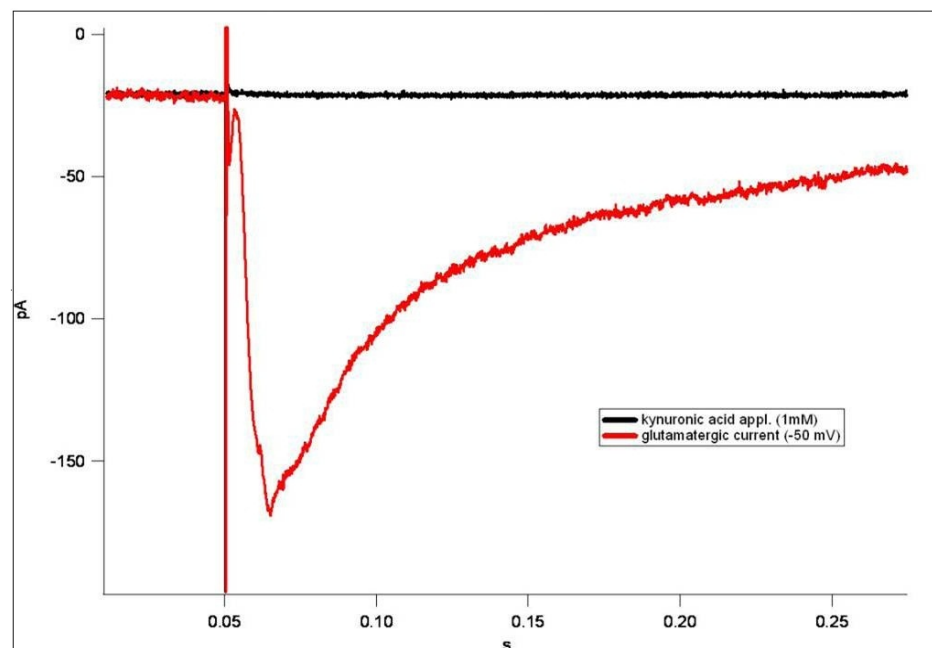
Figure 1.4 Whole glutamatergic current and its blockage. Glutamate response was totally blocked by kynuronic acid (1mM) application [22].

The NMDA receptor family is comprised of seven subunits, NR1, NR2A-D and NR3A, NR3B. AMPA receptors are composed of a four-subunit family, GluR1-4. Kainate receptors are divided into two related families, GluR5-7 and KA-1 and KA-2. iGluRs are composed of extracellular amino terminal domain, the second and third transmembrane domains are bonded by a large extracellular loop and the third

transmembrane domain forms an intracellular carboxy-terminus. The agonist binding domain is located between the extracellular N-terminal region (*S1 region*) and the extracellular loop between transmembrane domains 3 and 4 (*S2 region*).
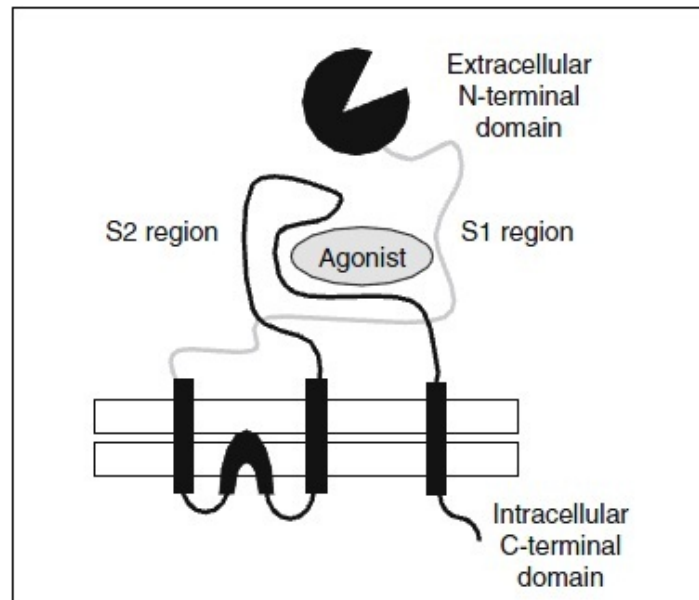
Figure 1.5 Figure of iGluR family member. The extracellular amino-terminal domain is linked to a first transmembrane domain. A pore forming membrane-residing domain follows the first transmembrane domain and it does not cross the membrane. After this, the second transmembrane domain is localized and linked the third transmembrane domain by a large extracellular loop. Finally, the third transmembrane domain is followed by an intracellular carboxy-terminus [21].

1.3.1.1 NMDA Receptors. The NMDA receptor is a one type of iGluRs. Glutamate activates the receptor by binding to the agonist site of the receptor as its ligand. After the activation of the receptor, it is occured that influx of Na^+ and small amounts of Ca^{2+} ions and efflux of K^+ ions.

NMDA receptors are heteromeric integral membrane proteins. These receptors are comprised of NR1 and NR2 subunits which have a functional and also modular structure. The NMDA receptors are unique ligand-gated ion channels in terms of the requirement for two obligatory co-agonists that are binding at the glycine and glutamate binding sites localized on the NR1 [23] and NR2 [24] subunits, respectively. Electrophysiological studies emphasized the requirement for the occupation of two independent glycine and two independent glutamate sites for the activation of NMDA

receptors [25]. Therefore, a functional NMDA receptor has to be comprised of two NR1 and two NR2 subunits as a tetramer. Different recognition sites are important for functional modulation of the receptor. 2-amino-5-phosphonopentanoic acid (AP5) and 3-[(±)-2-carboxypiperazin-4-yl] propyl-1-phosphonic acid (CPP) perform competitive type of modulation on glutamate binding site [26]. MK-801, ketamine, memantin, PCP, Mg^{2+} block the channel and perform non-competitive type of modulation. Co-agonist glycine is essential for the receptor activation [27]. Glycine binding site is blocked by kynuronic acid so that the receptor activity is inhibited [28].
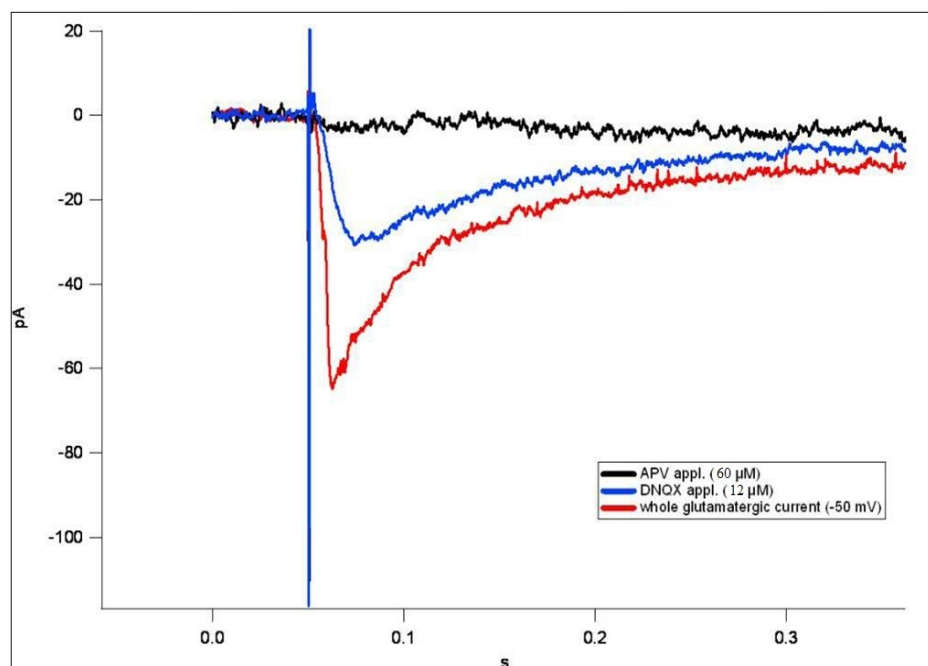
Figure 1.6 Isolation of NMDA current and its blockage. NMDA current was isolated from whole glutamate response by applying DNQX (12 μM). Additionally, AP5 (60 μM) which is an antagonist of NMDA blocked the whole NMDA current [22].

Magnesium ions have a voltage-dependent blocking effect on NMDA receptors so at the resting membrane potentials, NMDA receptors are inactive. If free Mg^{2+} concentrations in extracellular fluid is higher than normal level, transmission is depressed. The mechanism for this phenomenon is a competition between Mg^{2+} and Ca^{2+} because these ions are involved in the stimulus-secretion coupling processes in transmitter release [29]. Activation of NMDA receptors causes Ca^{2+} and Na^+ influx, and K^+ efflux.

NMDA receptors are significant for long-lasting modifications of synaptic strength

in critical neural circuits. Long-term potentiation (LTP) is one such activity-dependent modification in the hippocampus. In this concept, a sustained increase in synaptic strength is occured via brief high frequency stimulation of excitatory afferents. High frequency afferent activity generates the postsynaptic depolarization and during this period NMDA receptors are activated, which is a requirement for LTP generation [30]. As a result Ca^{2+} concentration is increased in the cell and this is a necessity for triggering the LTP. During low frequency synaptic transmission, the excitatory neurotransmitter, glutamate, is released from a presynaptic terminal. After secretion of glutamate, it binds to postsynaptic NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate (AMPA) receptors which are colocalized on a dendritic spine. The NMDA receptor isn't activated because Mg^{2+} ion blocks ion channel. On the other hand, the AMPA receptor provides synaptic responses at the resting membrane potential (-60 to -80 mV). When the postsynaptic membrane is depolarized while the generation of LTP, Mg^{2+} blockage is released and Ca^{2+}, Na^+ enter the cell. Sufficient Ca^{2+} ions enter the dendritic spine via repeated activation and this activates the signaling mechanisms for the generation of LTP.

1.3.1.2 Non-NMDA Receptors. AMPA and kainate receptors are subgroups of non-NMDA receptors. There is four-subunit family (GluR1-4) of AMPA receptors. It is thought that these receptors have a tetrameric structure [31]. The GluR2 subunit is very important to determine the permeability of heteromeric receptors to Ca^{2+}. Thus, AMPA receptors without GluR2 subunit are Ca^{2+}-permeable and also rectify inwardly which is the result of a voltage-dependent block of the ion channels [32]. In terms of the molecular determinant, the GluR2 receptors which are calcium impermeable have been classified as an arginine (R) at a critical site in the pore loop, m2 domain, whereas it is glutamine (Q) at the corresponding position in the other subunits [33]. Furthermore, GluR2 subunit is lacking in AMPA receptors which are located in the hippocampus and amygdala so their Ca^{2+} permeability is high and also they predominate in inhibitory interneurons. In contrast, pyramidal cells have enough GluR2 containing AMPA receptors so they are Ca^{2+}-impermeable [34].
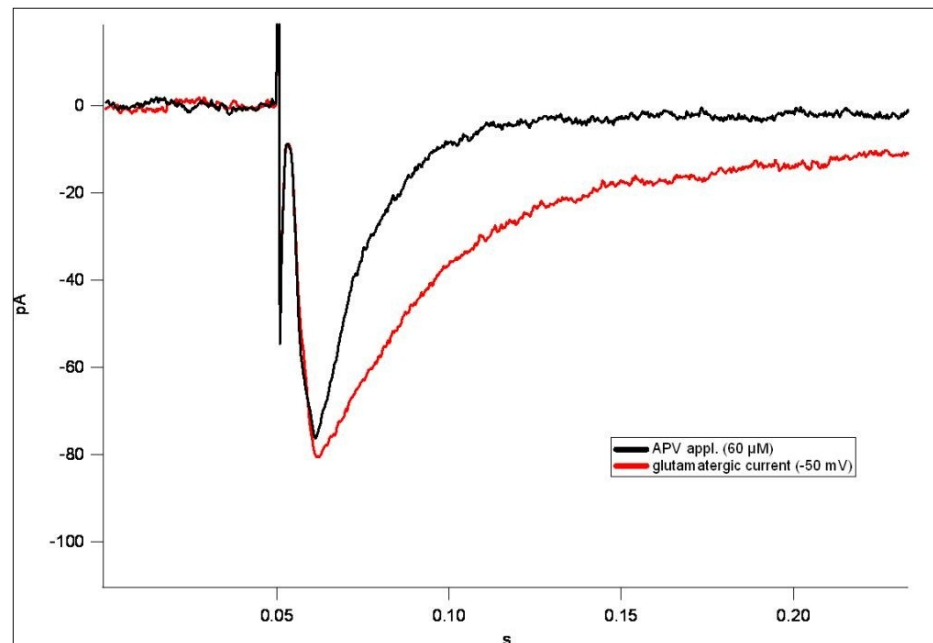
Figure 1.7 Isolation of non-NMDA currents. The antagonist of NMDA receptors, AP5 (60 μM) was used to isolate the non-NMDA current which is the fast component of glutamatergic current [22].

Kainate receptors are divided into two related subunit family, GluR5-7 and KA-1 and 2. Both homomeric and heteromeric combinations are possibly tetrameric. KA-1 and KA-2 subunits don't form functional homomeric receptors, however they form high affinity kainate binding sites and bind agonist ligands [21]. These two subunits assemble with members of the GluR5-7 subfamily to form functional heteromeric receptors [35]. It is observed that KA-2 subunit without GluR5-7 cannot be functional and it is retained in the endoplasmic reticulum [36]. Whereas, the GluR5-7 subunits can form functional homomeric receptors as well as form heteromeric receptors by combining with the KA-1 and 2 subunits via different pharmacological properties [37]. Kainate receptors have the same membrane topology with the other glutamate receptor subunits [38]. The amino terminal half of each subunit is extracellular and there are four hydrophobic segments. Three of them are membrane spanning domains and a "p-loop" which dips into the membrane from the cytoplasmic face to form the pore [39].

1.3.2 Metabotropic Glutamate Receptors

Metabotropic receptors are coupled to secondary messenger systems. These types of receptors are responsible for slower responses than ionotropic receptors do. Plus, metabotropic receptors do not generate significant changes in conductance and membrane potential [40]. The interaction of the metabotropic receptors control metabolic changes in the cells, like trophic activities. There are eight subgroups (mGluR1-8) of metabotropic glutamate receptor (G-protein-coupled) family which have been classified in terms of their sequence homology, second messenger coupling and pharmacology as three groups: group I (mGluR1 and 5), group II (mGluR2 and 3), and group III (mGluR4,6,7,8). Even though mGluR members can control synaptic transmission by activating slow excitatory postsynaptic transmission, they also carry out modulatory roles, such as regulating excitability, synaptic transmission and plasticity. mGluR-mediated signalling is demonstrated as a result of stimulation of second messenger systems, G-protein linked action and also activation of non-selective cationic channels [41].

All of the mGluR family members are observed in the mamalian CNS [42] except mGlu6 which is expressed in the retina [43]. Group I mGluRs are confined to postsynaptic part of somatodendritic domains, however group II and III receptors are localized presynaptically in axonal domains and axon terminals so they modulate neurotransmitter release [44].

mGluRs are subgroups of the G-protein coupled receptor (GPCR) family C (or 3) [45]. mGluRs have a large extracellular amino-terminal domain (ATD) [46] which is in bilobed configuration [47]. The ATD is connected to a GPCR transmembrane domain which controls the activation of G protein [45] and the C-terminus compartment regulates the receptor activity.

1.4   Cell Membrane and Sphingolipids

1.4.1   Cell Membrane

The first membrane model was proposed by Danielli and Davson in 1935. This model was called "sandwich model" and comprised of lipid bilayer covered on both sides with proteins. This model was used as the basic model for membrane structure by biologists for many years. In the early 1970s, the fluid mosaic model was substituted for the sandwich model. This model was proposed by S. J. Singer and G. L. Nicolson in 1972 and it admits that the basic lipid bilayer structure and beside this, the proteins are thought to be globular and to float within the lipid bilayer instead of forming the layers of the sandwich model [48]. Unwin and Henderson (1984) studied the nature of the membrane proteins. In accordance with their studies, the proteins which spread through the lipid bilayer is hydrophobic in nature and settled in a three-dimensional shape, in the form of an alpha-helix.
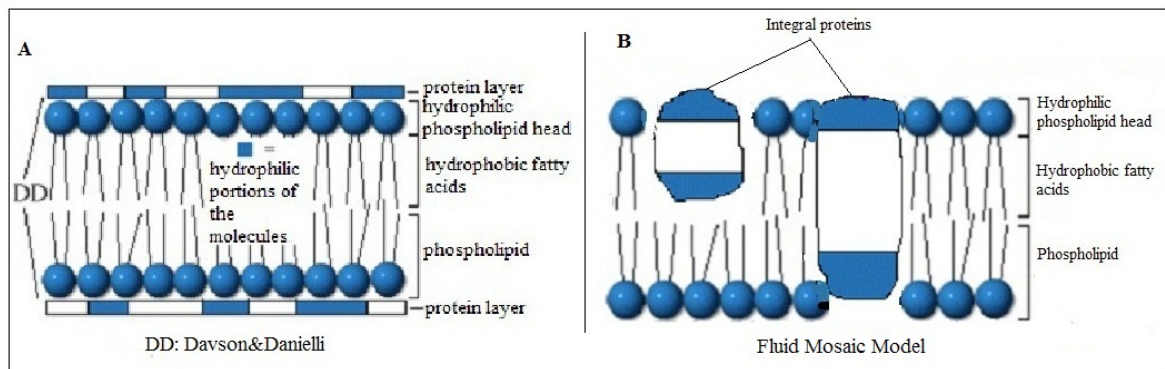
Figure 1.8   Cell membrane models. A. Davson-Danielli model which emphasize the lipid bilayer between two protein lines. B. Fluid mosaic model which propose the lipid bilayer with integral or peripheral proteins.

In the fluid bilayer concept, various lipid groups form a heterogenity in lateral dimension. Because of the dynamic structure of sphingolipids and cholesterol, they form rafts which shift within the fluid bilayer. By including or exluding the proteins from lipid microdomains or rafts, these microdomains play a role in transportion of specific membranes and signal transduction [49].
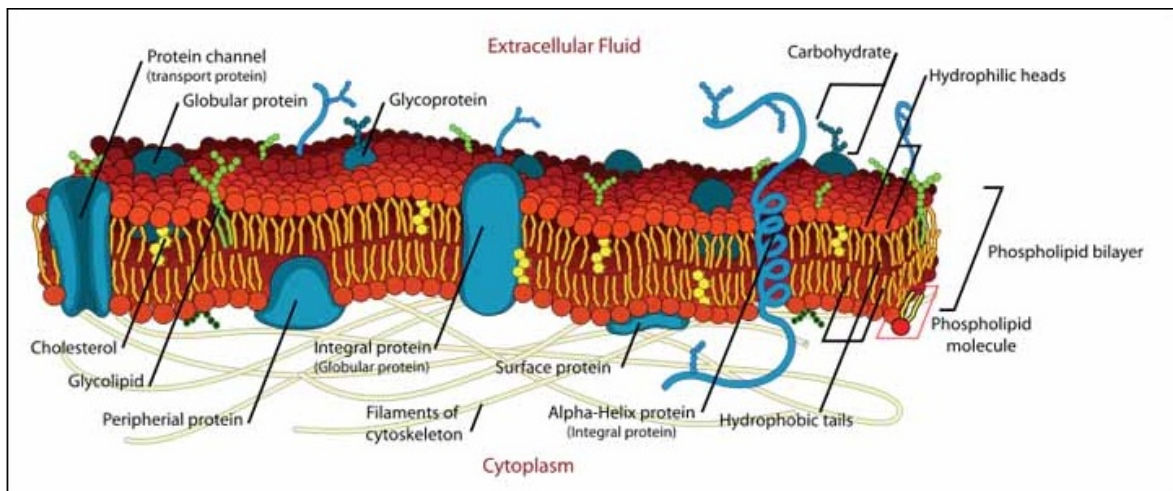
Figure 1.9 Fluid mosaic model with alpha-helix transmembrane proteins. The lipid bilayer exists in a fluid state and capable of dynamic movement. Membrane proteins penetrate the lipid bilayer to varying degrees.

1.4.2 Structure of Sphingolipids and Their Functions

A sphingoid base backbone forms sphingolipids. This backbone is synthesized *de novo* from serine and fatty acyl-coenzyme A and then changes form into complex compound such as ceramides, phophosphingolipids, glycosphingolipids and protein adducts (Fig 1.10) which are important for the structure of cell membrane (Fig. 1.9) [50]. Complex sphingolipids play key roles in cell growth, differentiation and migration as well as simpler sphingoid bases and derivatives. Sphingosine is a fundamental component of sphingolipids. Due to the fact that this molecule inhibits the function of protein kinase C [51], scientists hypothesized that sphingolipids have a function as second messengers in the cell [52]. This hypothesis was supported by the clarification of sphingomyelin (SM) cycle and the physiologic functions of ceramide [53].

1.4.3 Sphingolipids and Their Roles in Cell Metabolism

There are different sphingolipids with different structural features. Phosphosphingolipis and glycosphingolipids are complex sphingolipid members. Sphingomyelins are the major phophosphingolipids in mammals. On the other hand glycosphingolipids
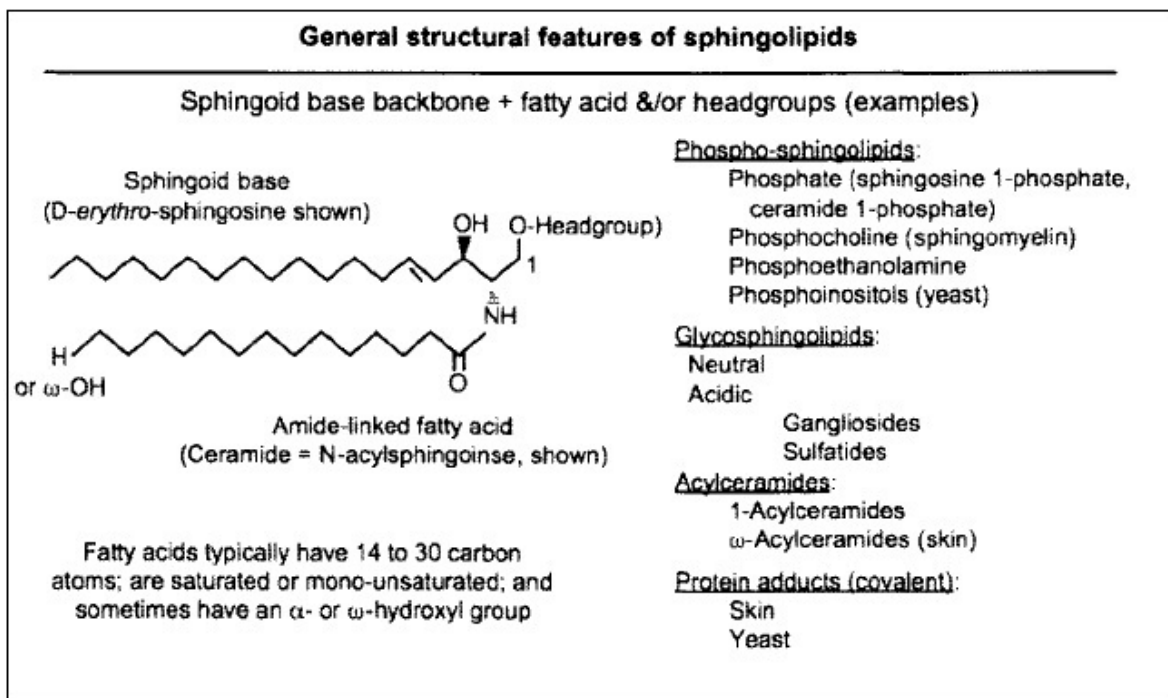
Figure 1.10 Overview of sphingolipid structures [50].

have one or more carbohydrate groups. Glycosphingolipids are basically categorized in terms of carbohydrates they are composed: 1) Neutral glycosphingolipids contain glucose as an uncharged sugar such as glucosylceramide (GlcCer), galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), and fucose (Fuc); 2) Acidic glycosphingolipids contain ionized functional groups (phosphate, sulfate) which bond to neutral sugars or charged sugar residues, sialic acid (N-acetylneuraminic acid, NeuAc) [50]. Moreover, some sphingolipids are bonded to protein covalently which are named protein adducts, for instance: ω-hydroxy-ceramides and -glucosylceramides are attached to surface protein of skin. The other member of sphingolipids is ceramide. A number of extracellular agents activate sphingomyelinase which cleaves membrane SM and concludes the process with the formation of ceramide. Ceramide mediates the effects of these agents on cell growth, differentiation and apoptosis.

In the late 1800s, the first sphingolipids were isolated and characterized as structural and inert component of cell membranes [54]. Furthermore, over the past two decades, it is known that beside their function in cell structure, lipids and lipid-derived

molecules have crucial functions in signal transduction and cell regulation. The structure of lipid-based signalling mechanisms can be rearranged. For instance, such module includes a regulated enzyme of lipid metabolism and specific targets that recognize the lipid which is formed. This modular structure is generally found in complex sphingolipids. Sphingolipids form a rich source of bioactive molecules. Many of the enzymes which are contained by sphingolipid metabolism and their products are involved in signal transduction and cell regulation [55].

Sphingolipid metabolites; ceramide (Cer), sphingosine (Sph), and sphingosine 1-phosphate (S1P), are known to function not only as intracellular second messengers, but also in the extracellular space. On the one hand, it has been suggested that Cer, Sph, S1P binds to specific intracellular protein targets as second messenger. On the other hand, there are different studies which focus on functions of these sphingolipid metabolites in the extracellular space. For instance, it is thought that S1P regulates cell motility and morphology as an extracellular messenger, beside its intracellular function, and also S1P is effective on various physiologic functions such as vascular maturation during development, heart rate, and lymphocyte recirculation [56].

Sphingolipids contain long, largely saturated acyl chains and by this way they diverge from most biological phospholipids [57]. The first steps of sphingoid base formation is followed by attachment of a fatty acid to form dihydroceramide. By inserting the cis 4-5 double bond, dihydroceramide is converted into ceramide. This molecule is the precursor for sphingomyelin and other complex sphingolipids [58]. In addition, Cer is known to function within the cell membrane Sphingomyelin (SM) degradation which is a major pathway involved in producing these lipid messengers. Signal-activated sphingomyelinases (SMases) break down the attachment between SM and the plasma membrane to form the Cer. The next step is proceeded by ceramidase (CDase). This enzyme converts the Cer into Sph. Subsequently, Sph is phosphorylated by Sph kinase into S1P in the cytoplasmic region [56].

Furthermore, the generation of second messenger, ceramide is ubiquitous and also evolutionary conserved [59]. Cer is an endogenous mediator of apoptosis as a result
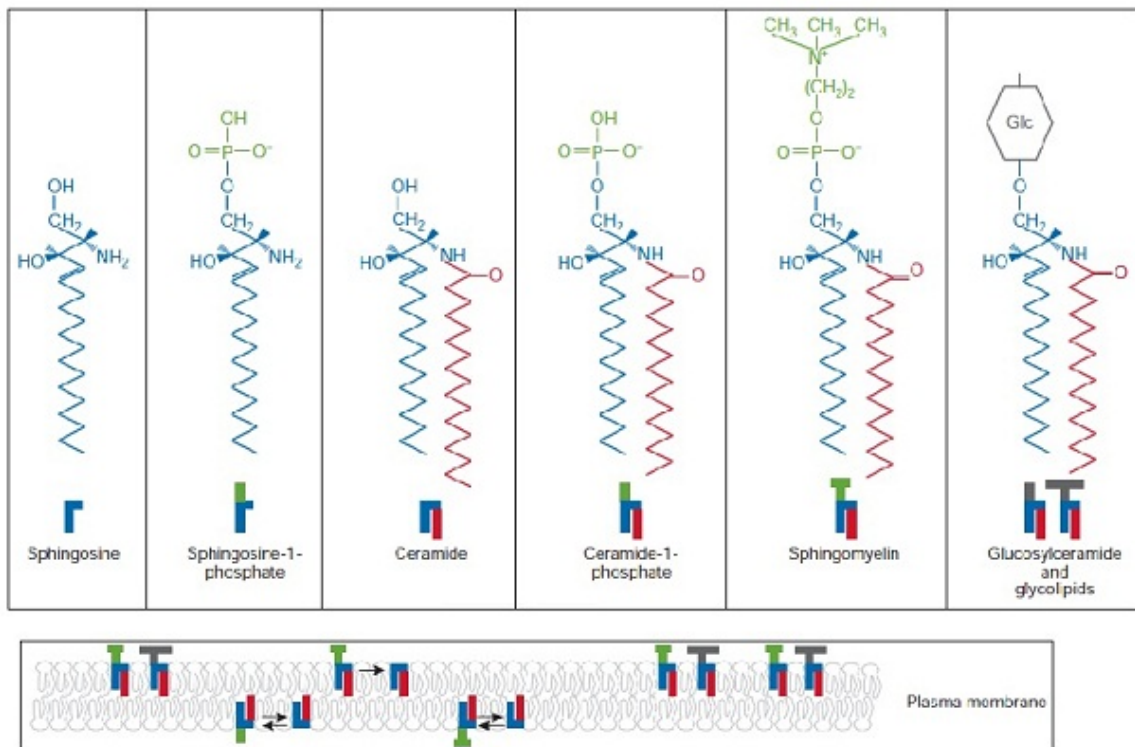
Figure 1.11 The complexity of sphingolipids. The structure of some sphingolipids are showed. There is only one type of sphingoid base which is sphingosine (in blue) and there is only one type of fatty acid which is palmitic acid (in red) in the figure. When these two components bond together, N-acylation is occured [58].

of cytokines, antigens, anticancer drugs, or environmental stress inducers. Moreover, via the accumulation of ceramide, induction of sphingomyelin hydrolysis is initiated by these stimuli. This process occurs before morphological changes. Furthermore, application of exogenous ceramide analogs cause apoptotic cell death. In this concept, scientists have proposed that ceramide could be effective in apoptosis signalling [60].

A lot of studies have been focused on sphingolipids during the past five years and many of them trying to elucidate the role of ceramide as a cellular bioregulator [55].

1.5 Ceramide

Ceramide is a sphingolipid that has important roles as a signaling molecule in vital processes such as apoptosis, growth arrest, senescense, differentiation, mediating an immune response, and cell cycle arrest [61]. Structurally, ceramides are comprised of a fatty acid chain, which are varied length, saturation, and hydroxylation, and this chain is bounded to the amino group of a sphingoid base via an amide linkage. Moreover, the hydrophobic backbone of all the complex sphingolipids such as sphingomyelin, cerebrosides, gangliosides is formed by ceramides. In terms of the length of fatty acid chain, ceramides are varied from two to 28 carbons. C-16 to C-24 ceramides are most abundant in mammalian cells. These fatty acids are generally saturated or monounsaturated, and they may contain a hydroxyl group at the C-2 position (α-hydroxy fatty acid) or on the terminal C atom (ω-hydroxy fatty acid) [62]. Ceramides are the most hydrophobic lipids in nature because of this, no ceramide exists in solution in cytosol. Thus, ceramides perform their primary actions only at the membrane level [63]. Free ceramides are found in large amounts in the skin stratum corneum, however they exist in smaller proportions in cell membranes where they are effective in cell signalling [57, 62, 63]. Additionally, their solubility in water is negligible, therefore this is another reason why they cannot exist in cytosol.

Ceramides can act only on membrane-related proteins which are linked to membranes. This may occur in two different ways. First, on proteins lacking specific ceramide binding sites, ceramides can induce changes in the physical properties of the membrane bilayer. For instance, increase in lipid chain order can cause changes in the enzyme activity. Both integral and intrinsic membrane proteins are affected by changes in bilayer order or fluidity [64]. Secondly, ceramides modulate enzyme action by binding to specific sites on the target protein. This mechanism can be performed on both permanent and transient membrane proteins. Two groups of proteins that are known to contain ceramide-binding sites can be distinguished: (a) Ceramide-binding membrane related proteins that are known to have at least transient connection in the membrane-bound form, such as protein kinase C, ceramide-activated protein kinase (CAPK), c-RAF-1. (b) Ceramide-binding proteins with no known membrane-binding

capacity. This is related with at least ceramide-activated protein phosphatases 1 and 2A (CAPP1 and CAPP2A) [63]. When ceramide molecules are included by phospholipid membranes as a part, the order of the acyl chains in the bilayer is increased by these molecules. This situation also causes lateral separation of domains enriched in ceramide. Thus, ceramide signaling occurs in mainly in localized domains in the cell plasma membrane. Moreover, ceramides permeabilize lipid bilayer which leads to vesicle fusion and fission processes (e.g., endocytosis).

There are several possible sources of ceramide that accumulates in response to extracellular stimuli. Mainly, sphingomyelinase and *de novo* pathways are studied. The other two ceramide formation pathways are salvage pathway and recycling of the exogenous ceramide.

1.5.1 The metabolic pathways for ceramide synthesis

There are four different metabolic pathways for ceramide formation [65]. The sphingomyelinase and *de novo* pathways are the major ones.
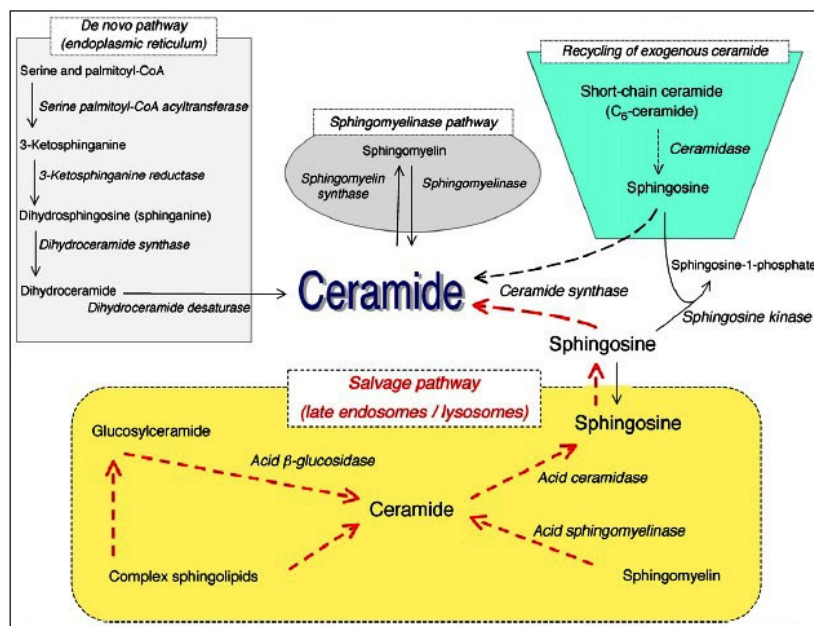
Figure 1.12 The metabolic pathways for ceramide synthesis; sphingomyelinase pathway, *de novo* pathway, exogenous ceramide-recycling pathway, and salvage pathway [65].

1. *The sphingomyelinase pathway*

   The sphingomyelinases are the most studied enzymes in sphingolipid mechanism. The neutral membrane-bound Mg^{2+}-independent sphingomyelinase (N-SMase) and lysosomal acid pH optima sphingomyelinase (A-SMase) are well-defined enzymes in terms of their roles in ceramide generation.

   Tumor necrosis factor-α (TNFα), Fas ligand, 1α,25-dihydroxyvitamin $D_3\gamma$-interferon, chemotherapeutic agents, heat stress, ischemia/reperfusion, and interleukin-1 are causes of increasing in N-SMase activity and a corresponding decrease in SM so an increase in ceramide level [66, 67]. Additionally, this enzyme is activated by both arachidonic acid and glutathione depletion [67, 68]. For instance, TNFα decreases glutathione and this leads to prolonged ceramide generation. Moreover, nerve growth factor (NGF) causes N-SMase-dependent cell death which is associated with Jun kinase (JNK) activation in hippocampal neurons [69]. Also, N-SMase activity is observed in cell membrane raft formation in neural cell apoptosis.

   Although the A-SMase is a lysosomal enzyme because of its optimum pH is 4.5-5.0, an isoform of this enzyme has been identified in vesicles near the plasma membrane and the isoform is secreted extracellularly [70]. In addition to this, a plasma membrane form of A-SMase has been defined in microdomains. Therefore, there may be three different types of A-SMase; acidic lysosomal A-SMase which is responsible for SM metabolism, secretory A-SMase which is important for inflammation and stress responses, and a receptor-activated A-SMase. The last form of A-SMase moves to the outer cell membrane as a result of its activation by various cell surface receptors such as CD95, CD40, and SM hydrolyzes into ceramide [71, 72, 73]. After releasing the ceramide molecule, it associates into the raft domains in the cell membrane and this association helps the clustering of activated receptor molecules [74]. Furthermore, ionizing-radiation-induced apoptosis is related with ceramide increase, even though it is not known in which sphingomyelinase enzyme involves.

2. *The de novo pathway*

   The *de novo* biosynthesis of ceramide occurs in the endoplasmic reticulum. This

pathway commences with the activation of serine palmitoyltransferase (SPT) which condenses serine and palmitoyl CoA to generate 3-ketodihydrosphingosine [65] that is reduced to form dihydrosphingosine (sphinganine). Dihydrosphinganine is acylated by dihydroceramide synthases to generate dihydroceramide [65, 70]. The acylated sphinganine is converted into the acylated sphingosine (ceramide) via addition of trans-4,5 double by dihydroceramide desaturase [54, 65]. Complex sphingolipids are formed from ceramide which is formed on the membranes of the endoplasmic reticulum and this biosynthesis continues in the Golgi apparatus. This reaction needs vesicular transport of ceramide to the Golgi apparatus and protein-facilitated transport by the ceramide transfer protein (CERT). After the formation of sphingolipids, they are distributed to plasma membrane and subcellular organelles and they enter a degradation and regeneration cycles [65]. Furthermore, addition of dihydroceramide does not induce apoptosis, it is thought that there is the requirement of desaturase enzyme activation in order to form ceramide in response to agonists [75]. As a result of TNF and chemotherapeutic agents, the *de novo* pathway is activated and this results in apoptosis as can be seen from nuclear morphology and DNA fragmentation [76]. CPT-11 (a camptothecin derivative), hexacylphosphocholine, daunorubicin, and etoposide are some examples of chemotherapeutic agents which activate *de novo* ceramide production [54]. Moreover, free palmitoyl CoA activates the *de novo* pathway for ceramide production. This argument has been implied that this pathway mediates the complications of diabetes and obesity which are caused by free fatty acids [77].

In extensive studies, short-chain ceramides (C2-ceramide and C6-ceramide) are used because of some reasons. Short-chain ceramides are cell membrane permeable and also water soluble. Therefore, they can be easily delivered to cell, unline natural long and long-chain ceramides [78]. These short-chain ceramides are required to examine the biological aspects of ceramide, to find the intracellular ceramide targets and different ceramide signaling pathways [79]. Besides, mammalian cells includes short-chain ceramides naturally. According to the most of the researchers, short-chain ceramides act as long-chain analogs, so short-chain

ceramides are often used in experiments.

1.5.2 Dihydroceramide: Inactive form of ceramide

Dihydroceramide is generated by acylation of sphinganine and then converted to ceramide by the addition of a 4,5-*trans*-double bond [80]. This conversion step is catalyzed by dihydroceramide desaturase due to the fact that dihydroceramide is less effective than ceramide in terms of bioactivity [75] so in *de novo* synthesis of sphingolipids, dihydroceramide plays a role as an intermediate [81]. Additionally, since the greater insolubility of the long-chain analogs of dihydroceramide, the *in vitro* activity is decreased when the chain length of amide-linked fatty acid of the dihyroceramide is increased [82].

2. MATERIALS AND METHODS

2.1 *In Vitro* Brain Slice Preparation

Sprague Dawley rats were used and decapitation was performed by guillotine. After removing the head skin, the skull was cut off and brain was removed quickly in less than 60 seconds. The brain was promptly transferred in to ice-cold carbogenated artificial cerebrospinal fluid (ACSF) which contains (mM): NaCl 125, KCl 3.75, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1.25, $NaHCO_3$ 26, D-glucose 10. The ACSF was aerated with carbogen (95% O_2, 5% CO_2), pH 7.4. The transversal hippocampal slices was obtained 200 μm thickness by using vibroslicer (Campden Instruments, UK). Slices were transferred into a pool filled with carbogenated ACSF at 36-38 °C. They were put on a grid and the aeration was applied from under the slices. Slices were incubated in this aeration chamber at least 45 minutes for equilibration. Then, slices were placed one by one into the recording chamber and they were perfused (2.0 ml/min) with carbogenated ACSF at room temperature. The experimental and surgical procedures were approved by the Animal Ethical Committee of Boğaziçi University.

2.2 Whole Cell Patch-Clamp Recording

The whole-cell patch-clamp recordings were taken from the soma of the CA1 pyramidal neurons. Patch-clamp amplifier (EPC-7, List Medicals, Darmstadt, Germany) were used for keeping the voltage at a fixed value and recording the respective current amplitude. A converter (ITC-18 A/D, Instrutech, Washington, USA) was used to filter the analog signals and then digitized. Strathcylde-WCP was used for data acquisition and MatLab was used to analyze the data. To generate constant current pulses, a stimulus isolation unit (Iso-Flex, A.M.P.I., Jerusalem, Israel) was used. Borosilicate recording electrodes (Hilfenberg, Malsfeld, Germany; Harward Apparatus, Massachusetts, USA) (pipette resistance = 4-7MΩ) were prepared by using a

pipet puller (PP-81, Narishige, Tokyo, Japan). Pipettes were filled with intracellular solution containing(mM): CsF 10.0, CsCl 40.0, EGTA 10.0, HEPES 10.0, CaCl2 1.0 (pH=7.4). Cesium (Cs^+) was used as the main cation to substitute K^+ ions and to block potassium conductance. Also, fluoride was used as the main anion to substitute Cl^- and block the chloride conductance. Intracellular fluid (ICF) was filtered 0.2 membrane filter in order to remove any particle in the solution and this solution was kept at -20°C as 0.5 ml aliquots. These aliquots were thawed for each experiment. The slices were first examined under the 4X objective to magnify the whole slice and to place the stimulus electrode on Schaffer collateral layer. After this adjustment, 60X water immersion objective was used to identify the pyramidal neurons from which the recordings were made.

Tungsten stimulus electrode was located on the Schaffer collateral layer to stimulate from the proximal apical dendritic synapses. Electrical stimuli was applied at a frequency of 0.1 Hz, the duration of stimulus was 0.5 ms.

Voltage pulses (4 mV) were applied to electrode to observe changes in the pipette resistance and capacitance through the oscilloscope while approaching to the pyramidal neuron. Ground electrodes were chlorinated before each experiments in order to prevent the noise.

Under 60X objective, the pyramidal neurons, their soma and dendrites were observed. When pipette was in the bath, the positive pressure was applied to the pipette through a syringe connected to recording electrode by a tubing in order to prevent the pipette tip clogging. The recording electrode was touched the neuron soma very gently. After that, when the decrease in current amplitude was observed on the oscilloscope screen, negative pressure was sligthly applied. Then, current amplitude was decreased to baseline level and capacitive currents were observed. By doing this, the resistance between the pipette tip and membrane was increased and gigaseal was formed between them. After the seal step, a further brief suction was applied and the cell membrane was broken so that the whole cell configuration was achieved. Fast and slow capacitive components were compensated by adjusting the knobes of the patch-
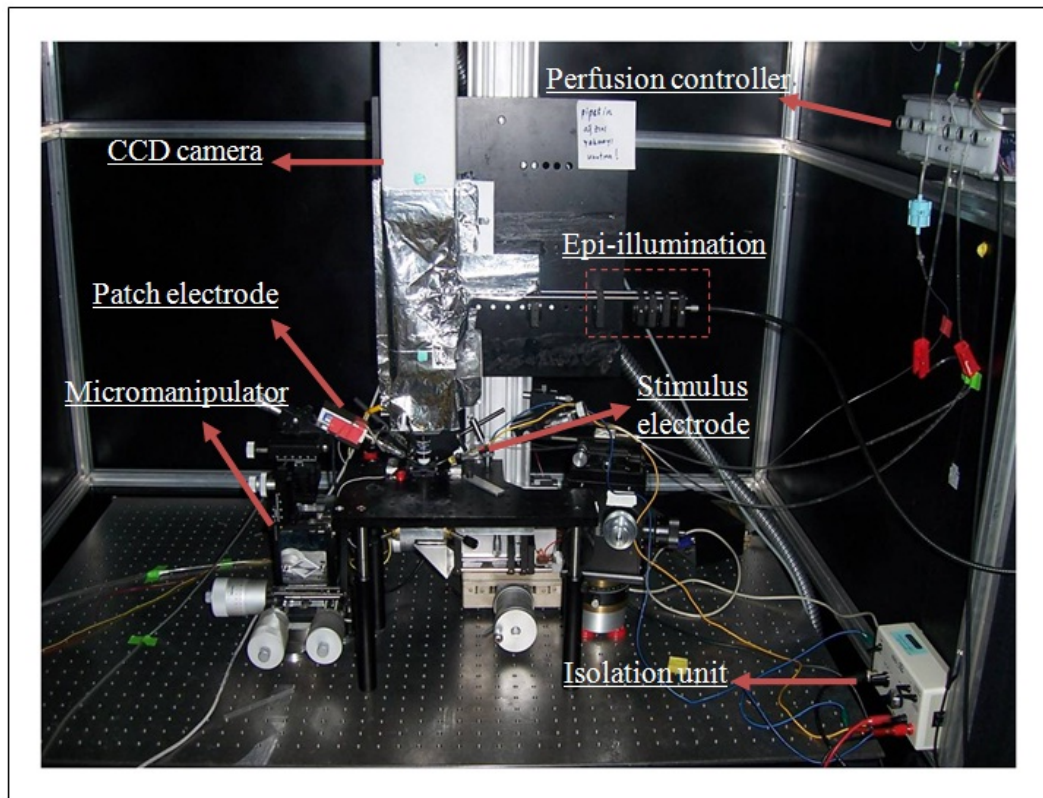
Figure 2.1 Setup for patch-clamp technique (Adapted from Genç Ö., MSc Thesis, 2007 [22]).

clamp amplifier. During the seal formation and break-in, the membrane potential was clamped at -60 mV (resting value), after the break-in, during the recording of membrane current the membrane potential was clamped at -50 mV (slightly depolarized). A current stimulus (0.5 ms) from stimulus generator was applied and excitatory post-synaptic currents (EPSCs) were recorded.

All the chemicals in ACSF were prepared before each experiments from the stock which was kept at +4°C. Kynuronic acid was used to block the glutamatergic response of the cell. By using this antagonist, we confirmed that we were working on glutamate receptors. 7-Dinitroquinoxaline-2,3-dione (DNQX) was used to antagonize the non-NMDA receptor mediated currents.

N-Acetyl-d-sphingosine (C2-Cer) was dissolved in dimethylsulfoxide (DMSO) and was kept 10 mM stock at -20°C. C2-Dihydroceramide was dissolved in hot DMSO and then it was kept as 10 mM stock at -20 °C as C2-ceramide. The final concentration

of DMSO during perfusion did not exceed 0.1 %. Sphingomyelinase was dissolved in phosphate buffered saline (PBS) (pH 7.4), and was prepared a stock of 1U/ml. Then, it was applied to the neurons (at different concentrations of 50 mU and 30 mU).

3. RESULTS

3.1 Whole Cell Patch-Clamp Records

3.1.1 C2-ceramide effect on NMDA currents

In the Fig 3.1, there are three different currents which were recorded from NMDA channels. Red current line was taken as a control group, blue current line represents the effect of C2-Cer $(10\mu M)$ application on NMDA current and green current line was recorded to see the effect of C2-Cer whether it was reversible or irreversible. There was a reducing effect of C2-Cer on NMDA current about 49.91%. The difference of these three currents is significantly different from each other (ANOVA, p=0.0081, α=0.05, n=6) indicating that C2-Cer had a significant depressing effect on NMDA currents recorded from CA1 pyramidal neuron of hippocampus which was irreversable due to the fact that the green current did not return to the level of red current.

We also analysed the differences of current means $(\mu_{\Delta I})$ (Table 3.1) for this experimental group. The differences between the control current and application current $(\mu_{\Delta I_{A1}})$, application current and wash current $(\mu_{\Delta I_{A2}})$, control current and wash current $(\mu_{\Delta I_{A3}})$ were studied. In accordance with this grouping, the difference between NMDA current (red line) and NMDA current with C2-Cer (blue line) was significant as a result of paired t-test. There was a significant difference between NMDA current and NMDA current after the application of C2-Cer (green line), as the difference of control and application current. However, the difference between NMDA current with C2-Cer (application current) and NMDA current after the application of C2-Cer (wash current) was not statistically significant. In short, we can reject the null hypothesis that the difference of current means is zero for the first and the second samples with probabilities 0.009 and 0.0212, respectively. For the last sample (application current-wash current), we can accept the null hypothesis with a probability of 0.7879. According to these results, there are three points need to be emphasized. First of all, since $\mu_{\Delta I_{A1}}$
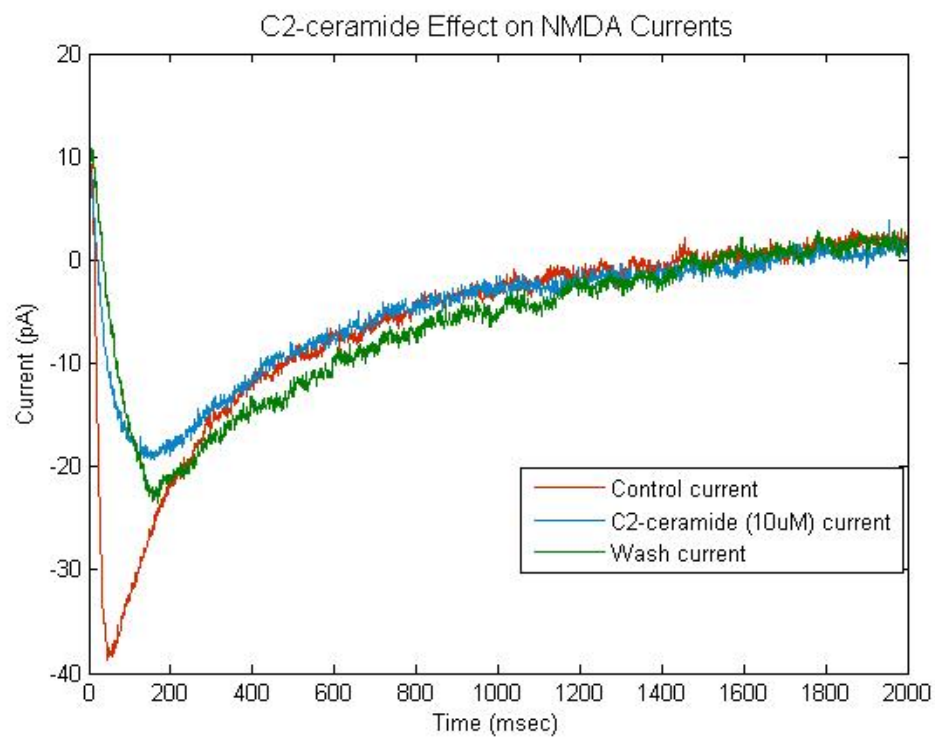
Figure 3.1 C2-ceramide (C2-Cer) effect on NMDA currents. Red current line shows the NMDA current in control conditions. DNQX (7-Dinitroquinoxaline-2,3-dione) (12 μM) was used to isolate the NMDA current from whole glutamatergic current. Blue current line shows the NMDA current which was treated with C2-ceramide (10 μM). Green current line shows the NMDA current washed with ACSF in the presence of DNQX (12 μM). (Whole records were taken under the condition of DNQX (12 μM) because of its reversible blocking effect on non-NMDA channels.)

Table 3.1
Troughout this chapter, the following abbreviations were used for statistical explanations.

Definition	Overall	C2-Cer	C2-DHCer	SMase(30mU)	SMase(50mU)
the current means	μ_I	-	-	-	-
the differences of current means	$\mu_{\Delta I}$	-	-	-	-
the difference of current means between control-application	-	$\mu_{\Delta I_{A1}}$	$\mu_{\Delta I_{B1}}$	$\mu_{\Delta I_{C1}}$	$\mu_{\Delta I_{D1}}$
the difference of current means between application-wash	-	$\mu_{\Delta I_{A2}}$	$\mu_{\Delta I_{B2}}$	$\mu_{\Delta I_{C2}}$	$\mu_{\Delta I_{D2}}$
the difference of current means between control-wash	-	$\mu_{\Delta I_{A3}}$	$\mu_{\Delta I_{B3}}$	$\mu_{\Delta I_{C3}}$	$\mu_{\Delta I_{D3}}$

was statistically significant, it was shown that C2-Cer application decreased the current through the NMDA channels. Second, C2-Cer had an irreversable effect on this current because $\mu_{\Delta I_{A3}}$ was statistically significant. Third, although there was an insignificant difference between C2-Cer applied NMDA current and wash current, this fact showed that the effect of C2-Cer was persistent on NMDA channels which implied the second point.

3.1.2 C2-dihyroceramide effect on NMDA currents

In the Fig 3.2, there are three different currents which were recorded from NMDA channels. It appeared that C2-DHCer decreased the amplitude of NMDA current about 48.47%. Statistically, these three currents were significantly different from each other (ANOVA, p=0.006, α=0.05, n=9), so it can be said that there was a significant reducing effect of C2-DHCer and also this effect was irreversable due to the fact that the wash current did not return to the level of control current.

Moreover, to understand the difference of current means, the difference between control current and application current ($\mu_{\Delta I_{B1}}$), application current and wash current ($\mu_{\Delta I_{B2}}$), control current and wash current ($\mu_{\Delta I_{B3}}$) were studied by using the paired t-test. In accordance with this grouping, it was possible to reject the null hypothesis, which the difference of current means was zero for all three samples. This argument means that the $\mu_{\Delta I_{B1}}$ is significant therefore it can be said that C2-DHCer had a
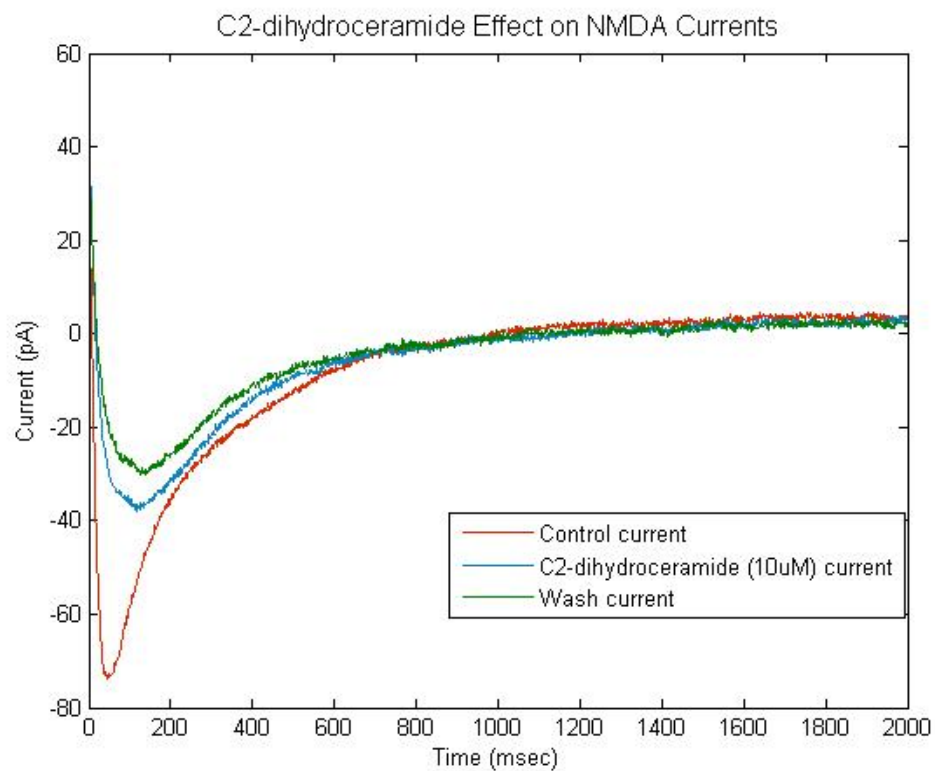
Figure 3.2 C2-dihyroceramide (C2-DHCer) effect on NMDA currents. Red current line shows the NMDA current under control conditions. DNQX (7-Dinitroquinoxaline-2,3-dione) (12 μM) was used to isolate the NMDA current from whole glutamatergic current. Blue current line shows the NMDA current which was treated with C2-DHCer (10 μM). Green current line shows the NMDA current after the application of C2-DHCer, in control conditions with DNQX (12 μM) (wash). All recordings were made in the presence of DNQX(12 μM) in ACSF to isolate the NMDA current.

reducing effect on NMDA current as C2-Cer. Additionally, the significant difference between NMDA current with C2-DHCer and NMDA current after the application of C2-DHCer showed that C2-DHCer had an irreversible-decreasing effect on the NMDA current. Besides, the significant difference of control current and wash current implied the irreversible effect of C2-DHCer on NMDA current.

3.1.3 SMase (30mU) effect on NMDA currents

In the Fig 3.3, there are three different currents which were recorded from NMDA channels. It appeared that SMase decreased the amplitude of NMDA current about 32.14%. Statistically, these three currents were not significantly different from each other (ANOVA, p=0.0618, α=0.05, n=3), which might be explained by the small sample size.
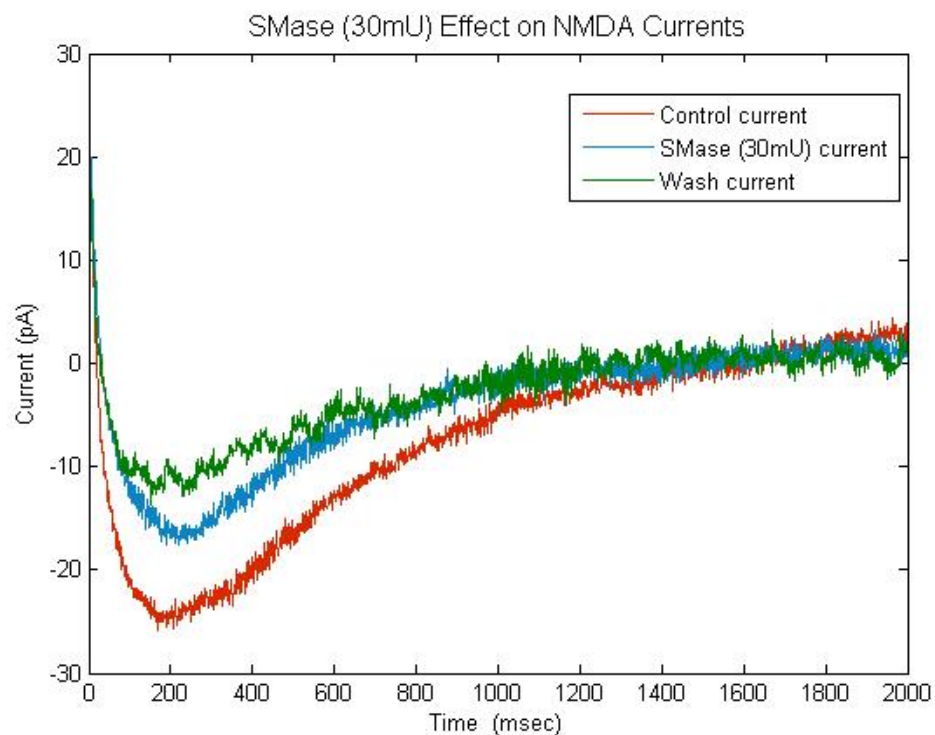
Figure 3.3 Sphingomyelinase (SMase) effect on NMDA currents. Red current line shows the NMDA current in control conditions. DNQX (7-Dinitroquinoxaline-2,3-dione) (12 μM) was used to isolate the NMDA current from whole glutamatergic current. Blue current line shows the NMDA current which was treated with SMase (30mU). Green current line shows the NMDA current during the wash of Smase (30mU), in the presence DNQX (12 μM).

On the other hand, the difference of current means were studied by using the paired t-test as for C2-Cer and C2-DHCer. According to the grouping, the only significant difference was determined between NMDA current (control) and NMDA current with Smase (30mU) (p=0.0136). Although the sample size was small, the enzyme was strongly effective on NMDA current. Whereas, the other two differences of current means (the application current-the wash current ($\mu_{\Delta I_{C2}}$), the control current-the wash current ($\mu_{\Delta I_{C3}}$)) were not significantly different (p=0.3116 and p=0.0606, respectively).

3.1.4 SMase (50mU) effect on NMDA currents

As shown in Fig 3.4, it appeared that the amplitude of NMDA current was decreased about 62.66% by SMase (50mU). Statistically, these three currents were significantly different from each other (ANOVA, p=0.0242, α=0.05, n=9) so that there was a significant reducing effect of SMase (50mU) and also this effect was irreversable due to the fact that the green current did not return to the level of control current.

In addition, the significance of current means differences were examined by using paired t-test. The difference between control and application current ($\mu_{\Delta I_{D1}}$), application current and wash current ($\mu_{\Delta I_{D2}}$), control current and wash current ($\mu_{\Delta I_{D3}}$) were the groups tested. As a result, the difference between NMDA current (control current) and NMDA current with SMase (application current) was significant. There was a significant difference between NMDA current and NMDA current after the application of SMase (50mU) (wash current). However $\mu_{\Delta I_{D2}}$ was not statistically significant. In short, we can reject the null hypothesis that the difference of current means is zero for the first and the second samples by the probability of 0.0073 and 0.0103, respectively. We accept the null hypothesis for the last sample (application current-wash current) by the probability of 0.4545. According to these results, there are three points need to be emphasized. First, we showed that SMase (50mU) application decreased the current amplitude of NMDA channels. Second, SMase (50mU) had an irreversable effect on this current because $\mu_{\Delta I_{D3}}$ was significant. Third, insignificant difference between SMase (50mU) applied NMDA current and wash current supported that the effect of
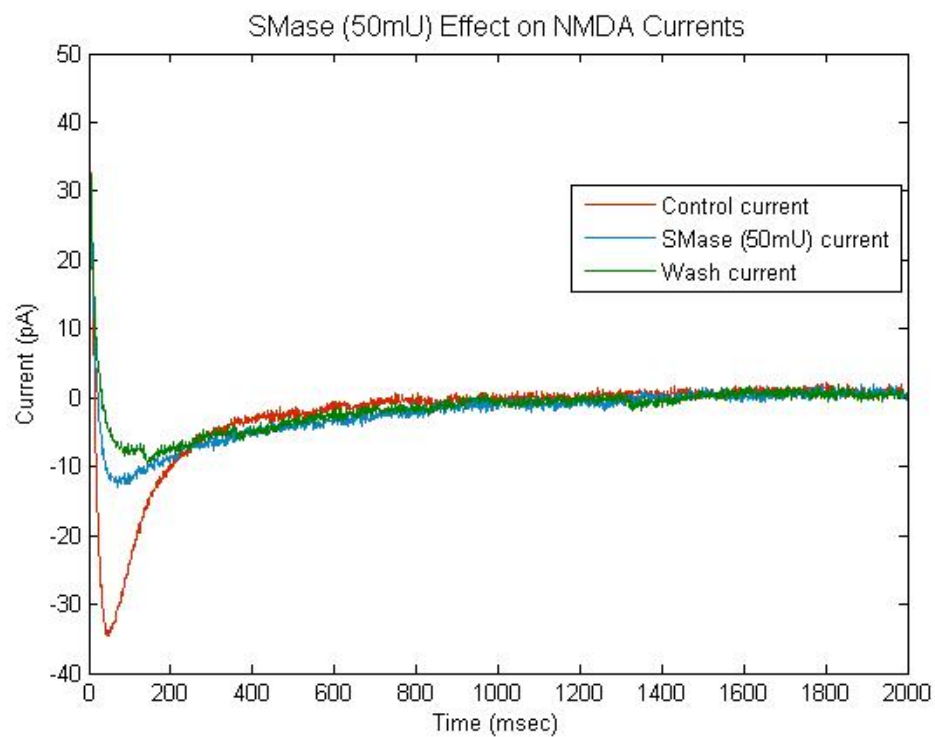
Figure 3.4 Sphingomyelinase (SMase) effect on NMDA currents. Red current line shows the NMDA current in control conditions. DNQX (7-Dinitroquinoxaline-2,3-dione) (12 μM) was used to isolate the NMDA current from whole glutamatergic current. Blue current line shows the NMDA current which was treated with SMase (50mU). Green current line shows the NMDA current after the application of Smase (50mU), under control conditions with DNQX (12 μM). (Whole records were taken in the condition of DNQX (12 μM) because of its reversible effect on non-NMDA channels.)

SMase (50mU) was persistent on NMDA channels which implied the second point.

3.1.5 Overall view of the effects of chemicals on NMDA currents
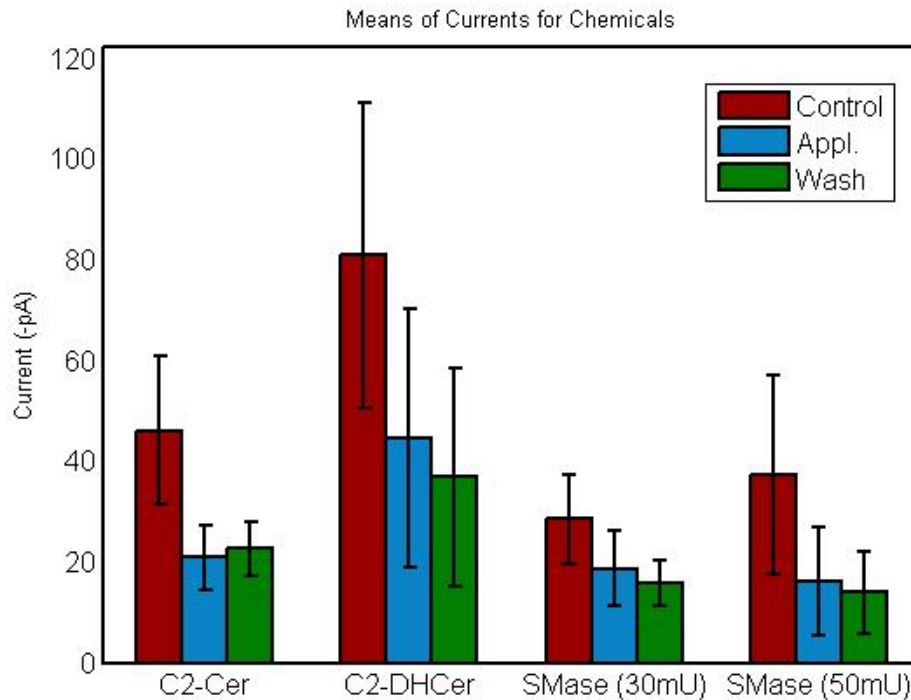
Figure 3.5 Means of currents for chemicals. There are 4 different chemicals applied to the NMDA current and the amplitudes of the NMDA currents with $\mu \pm \sigma$. Red bars indicate means of the control currents which were recorded from NMDA channels in the presence of DNQX. Blue bars show the means of the NMDA current treated with both DNQX (because of blocking the non-NMDA channels) and chemicals (application currents). Green bars represent the means of NMDA current after the application (wash currents).

As can be seen from the bar charts, all of the chemicals have a reducing effect on NMDA current. Also these effects are irreversible.

In addition to ANOVA and paired t-test, Holm-Sidak test was used to analyze the means of current difference between control currents and application currents for all four groups. The null hypothesis (H_0) was that there was no difference between the pairwise means of current difference for four application groups. When the uncorrected p values of pairwise comparison groups were ordered, the smallest p value was found 0.0562 from the couple of C2-DHCer and SMase (30mU). Uncorrected p value was bigger than the computed p value which was 0.0085 so that there was no significant

Table 3.2
p values of paired t test for all applications. According to these results the significancy of the current differences were determined.

p values of paired t-test (α=0.05)			
Chemicals	**Control-Appl.**	**Appl.-Wash**	**Control-Wash**
C2-Cer	0.009	0.7879	0.0212
C2-DHCer	0.003	0.0270	0.0016
SMase (30mU)	0.0136	0.3116	0.0606
SMase (50mU)	0.0073	0.4545	0.0102

difference between the C2-DHCer and SMase (30mU) pairwise, in terms of means of current difference between control and application currents. Due to the fact that the first value of the Holm-Sidak test was failed to reject the H_0, the remained pairwise comparisons weren't analyzed and H_0 was accepted for all five paired groups so there was not significant difference between them, as well, in terms of the means of current difference between control and application currents.

4. DISCUSSION

In our research, we used patch-clamp technique and there are several different parameters of electrophysiological response that can be recorded from *in vitro* rat brain hippocampal slices. According to the tri-synaptic circuitry, the Schaffer collateral is the link which provides a connection between CA3 and CA1 region of hippocampus and it is the most convenient area to stimulate the pyramidal neurons of CA1 region. There are two types of burst discharges in neuronal system. These discharge types are termed *endogenous burst* and *network-driven burst*. Even though they are mechanistically different, frequently similar in appereance. The currents which maintain an endogenous burst are independent of other cell activity and are produced within a single cell. On the other hand, the currents which maintain a network-driven burst are dependent on a group of neurons so the synaptic interaction among cells are important for this type of burst discharge. Moreover, the frequency of endogenous bursts is voltage dependent and this type of burst is not related with synchronized activity of neurons. In contrast, it has been reported the voltage independency of the network-driven burst and also in that records, synchronized rhythmic cellular activities have been determined. Both CA3 and CA1 neurons display not only endogenous bursts but also network-driven bursts. Nevertheless, the network-driven burst is sustained by synaptic currents which are produced by recurrent excitatory circuitry and this circuitry is observed in CA3 region but not in CA1 [83, 84]. Furthermore, the recurrent excitation of CA3 region causes some epileptiform activities, which is not suitable for our investigation. That was the reason of our work on CA1 region of hippocampus. For evoking synaptic responses, a bipolar tungsten stimulating electrode was placed in stratum radiatum, and Schaffer collateral/commissural fiber were stimulated at 0.1 Hz because this frequency is close to the physiologic conditions. We kept the membrane holding potential at -50mV and the potential difference of stimulus is 2-5mV. Under these conditions, stimulating the Schaffer collateral pathway was sufficient to obtain the glutamatergic response. The distance between the stimulus electrode and the pyramidal neuron which we patched was also very important. It affected the amplitude of the neuronal response so we

located it 90-100 μm away from the target pyramidal neuron.

Glutamate is a crucial and a universal neurotransmitter for neural system. After stimulation, glutamate mediated the synaptic transmission to form excitatory post synaptic current (EPSC). To distinguish the EPSC components, which are NMDA and non-NMDA receptors, DNQX was used (an antagonist of non-NMDA receptors). By doing this, NMDA receptors were isolated and after the isolation of NMDA receptors, we used kynurenic acid which is a global antagonist of iGluRs. Therefore, we confirmed that we were working on NMDA receptor which is a sub-component of iGluR.

4.1   Applications on NMDA receptors

In this study, we have focused on active and inactive forms of sphingolipids, and also their enzyme, SMase. Sphingolipids are not only structural component of cell membranes but also they are functional on signal transduction and cell regulation [53]. Ceramide which is one type of sphingolipids has important roles as a signaling molecule in determining the fate of cells such as apoptosis or differentiation [60]. As a second messenger, ceramide is increased by TNFα, Fas ligand, heat stress, chemotherapeutic agents, ionizing radiation via activating the SMase. Ceramide modulates some enzyme action such as kinase and phophatase by binding to specific sites on them [62]. These enzymes have effects on some membrane proteins, such as ion channels. Besides, it has been reported that ceramide mediates the ion flux. It inhibits the voltage dependent K$^+$ channels (Kv 1.3) which causes apoptosis [85]. Moreover, CD95 regulates the Ca release-activated Ca channels (CRAC) and the inhibitory effect of CD95 on CRAC is mediated by ceramide and A-SMase [86]. The intracellular functions of ceramide as a second messenger are still being worked in various studies.

1. *C2-ceramide effect on NMDA currents* In our investigation, we have taken our records for 15 minutes; each 3 phases of records being 5 minutes long. We examined the role of C2-ceramide (10μM) on the NMDA currents, according to our results, there was a depressing effect on NMDA currents about 49.91% at

the end of 5 minutes application. In order to check the reversibility of the response reduction, we washed the slices with ACSF and as a result, the current didn't reach to its initial level (5 minutes). ATP was not used in ICF to focus on membrane modulation because of the absence of ATP, we excluded the contribution of intracelullar part of neurons. It was previously reported that ceramide activated protein phosphatases and the sustained depression of synaptic EPSCs was mediated by the activation of postsynaptic protein phosphatases [87]. Moreover, it was shown that the extracellular application of ceramide can effect the intracellular cascades [88]. In our experiments, we blocked the intracellular mechanisms and applied the ceramide extracellularly, therefore we can say that ceramide not only triggers the intracellular mechanism but also modulates the membrane proteins extracellularly. This modulation can occur by binding to NMDA receptor sites. There are specific modulatory binding sites for Mg^{2+}, phencyclidine (PCP), polyamines, and Zn^{2+} [89]. The Mg^{2+} and PCP sites are located inside the ion channel, while the glycine, glutamate and polyamine binding sites are found outside the ion channel. Apart from these, arachidonic acid and ethanol have a modulatory effect on NMDA channel [90, 91]. Extracellularly applied ceramide can act as an antagonist of these binding sites and block the NMDA channel chemically. Besides chemical blockage, there can be a physical obstruction through the ion channel caused by ceramide molecules. Moreover, there are different point of views about the extracellularly addition of C2-Cer of C6-Cer to cells. According to some researches, both analogs are able to increase endogenous ceramide levels, even though it was thought that short-chain analogs trigger the ceramide formation instead of mimicking the effect of it [78]. Nevertheless, our investigation didn't support this idea because the intracellular mechanisms were blocked by the lack of ATP.

According to the result of wash, we can suggest that C2-ceramide has an irreversible depressing effect on NMDA receptors because the wash current didn't turn the initial level. It kept the stability around -23 pA so there was no apoptotic effect of 10 μM C2-Cer application and the pyramidal neuron was alive at the end of the record. It has been reported that 10 μM C2-Cer had an irreversible

effect on oligodendrocyte cell line, and this effect was apoptotic [92].

Collectively, the long-term depressed modulation of ceramide on NMDA receptor-mediated functions can be important in different physiological and pathological conditions in which the ceramide signaling pathway is activated.

2. *C2-dihydroceramide effect on NMDA currents* Due to the fact that C2-DHCer is inactive form of C2-Cer, in several studies it has been reported that there was no significant effect of this inactive analog [53]. However, in our experiments we observed that C2-DHCer depressed the NMDA receptor response by 48.47%. According to the result of wash process, the response didn't reach to its initial level. ATP conditions were similar to the group of C2-Cer application so we worked on only membrane channels and blocked the effect of the intracellular region of neurons.

It was reported that dihydroceramide is inactive in differentiation, antiproliferation and apoptosis, unlike ceramide [53]. Dihydroceramide lacks the 4,5-trans-double bond however it keeps the stereochemical configuration of ceramide. Also its uptake and metabolism are very similar to those of ceramide [93]. Some researchers reported that as a result of the specificity of ceramide action, introduction of the double bond is significant for imparting the biochemical and biological activity of ceramide [75]. Therefore, for instance, the ability of ceramide to induce apoptosis is because of specific structural requirements but not nonspecific hydrophobic interactions [94]. According to these, Triola et al. emphasized that the 4,5-trans-double bond contributes to the signaling function of ceramide and its metabolites [80]. Moreover, the lack of activity of DHCer arises from the lack of activity of DHCer on CAPP [53]. Bielawska et al. have found that ceramide inhibits the growth of yeast and activates yeast CAPP, where dihydroceramide is inactive. They have concluded that sphingoid backbone is inactive and introduction of either a double bond or a hydroxyl group forms an active molecule [75]. They also added that, there can be important physiological consequences because of the lack of dihydroceramide. DHCer is biologically inert so it would not be detrimental to cell viability and regulation of growth. The introduction of the double bond occurs after the formation of DHCer and this delay in introducing

the double bond may be a protection of cells from these regulatory functions [75].

Tepper et al. applied C2-DHCer extracellularly and observed that viabilities did not decrease significantly [66], in contrast ceramide was accompanied by significant cytotoxicity [54]. Furthermore, it was thought that ceramide induced pore formation. Siskind and Colombini found that C2 and C16-Cer form pores in phospholipid bilayer, however the capacity of the channel forming is limited for DHCer [95]. This was a different finding from previous studies because dihydroceramide was also inactive and did not mimic the effects of ceramide [82]. Plus, Goni and Alonso reported that DHCer is active to some extent in permeabilizing planar lipid bilayers [96]. Also, protein kinase C (PKC) has been implicated that it was one of the important components of signaling pathways regulating cell growth. Even though C2- and C6-Cer inhibit the PKC activity, C2- and C6-DHCer slightly stimulate the PKC activity [97]. Chik et al. have worked on ceramide effect on Ca^{2+} channel current in rat pinealocytes and they have found that C2 and C6-Cer was effective in reducing the increase in $[Ca]_i$ caused by BayK8644 whereas C2-DHCer was ineffective [98]. Additionally, Cer and C2-DHCer both increased steroid hormone production in MA-10 Leydig cells, but C2-DHCer was less effective than Cer. C2-DHCer concentration should be higher than Cer to mediate the same effect of Cer. Several previous studies have shown that C2-DHCer is inactive in apoptosis or induction of cell death. However, other investigators have found that C2-DHCer have some effect (albeit reducing effect) in assay systems [99].

In our investigation, we have found a strong depressing effect of C2-DHCer on the NMDA current. Nevertheless, we worked on only membrane proteins so we can not speculate on any intracellular effect (CAPP, PKC). Beside the chemical effect of C2-DHCer, we considered some physical features of C2-DHCer which caused a depression on NMDA current. It is believed that DHCer is inactive form of Cer and has no or little effect on cell viabilities. However, we found that DHCer has almost the same effect on NMDA current and it was shown that the neurons were still alive at the end of the records. Thus, we can say that 10 μM extracellular C2-DHCer application did not cause cell death but induced a strong

depression on NMDA currents and the physical attachment might be the main reason of this effect.

3. *Sphingomyelinase effect on NMDA currents* So far, we applied the Cer molecule extracellularly by using the perfusion system. Besides these applications, to find the effect of SMase, which is an enzyme for ceramide synthesis, this enzyme was applied to pyramidal neuron. Two different concentrations of SMase were used; 30 mU/ml and 50 mU/ml, according to the results, both of them caused depression on NMDA channels by 32.14 % and 62.66 %, respectively.

Statistical analysis showed us that exogenous application of N-SMase (50 mU) generated the same effect on NMDA currents as exogenous application of Cer. Chik et al. have reported that exogenous SMase treatment, which induces the hydrolysis of sphingomyelin, has the same effect as C2- or C6-Cer [98]. According to their results, they have suggested that C2- and C6-Cer, the synthetic ceramides, mimic the effect of endogenously produced ceramide, generated in the sphingomyelin cycle [98]. However they have used 5 mM ATP-Na$_2$ in intracellular fluid and it is different from our ICF composition but the results were similar. Besides the addition of ATP, they have applied 100 mU/ml SMase for 25 minutes however, in our case we applied 50 mU/ml SMase for only 5 minutes. Therefore, the inclusion of ATP in ICF is not enough to explain the difference for 5 minutes application between their and our investigation. Furthermore, it has been found that also SMase increased steroid hormone production as well as Cer [99]. Additionally, inhibition of N-SMase is crucial to suppress neuronal death from ischemia [100]. Wheeler et al. have pointed out that pharmacological inhibition of N-SMase prevents TNFα-induced generation of ceramide, phosphorylation of NR1 subunits of NMDA receptors, clustering of NR1, enhancement of NMDA-evoked calcium flux and EPSCs [101]. Recent studies have also supported that N-SMase plays an important role in membrane trafficking [102]. It was pointed out that N-SMase is an intermediate step of the TNF-α-induced clustering of NMDA receptors in lipid rafts of hippocampal neurons. Milhas and Hannun have recently found that even though N-SMase may traffic from the plasma membrane to the recycling endosomes, the budding of exosome vesicles

were determined in endosome-like domain of the plasma membrane. Therefore, N-SMase can be transferred into the cell via exosome biogenesis from the plasma membrane. It is possible that extracellularly applied N-SMase can be activate the formation of ceramide by exosomal transfer.

Furthermore, Norman et al. have applied N-SMase to cultured cells to record the effect on population spike (PS) amplitude at CA1 synapses [103]. They have performed field potential recordings from CA1 striatum radiatum and CA1 stratum pyramidal layers. It was reported that after the application of SMase the PS was increased but field excitatory post-synaptic potential (fEPSP) was unchanged. According to Norman's group, at the end of 30 minutes washout after 15 minutes N-SMase application, the PS returned the baseline so the effect of N-SMase was reversible on the PS at CA1 neurons. However, in our investigation, we found that at the end of 5 minutes wash, the response didn't recover to baseline so it seemed that the effect of N-SMase on NMDA current is irreversible. Also, Norman's group has investigated brief application of N-SMase on LTP and they have found that brief exposure of N-SMase to hippocampal slices didn't change LTP expression. Moreover, they showed that N-SMase increased the hippocampal neuron excitability via increasing action potential (AP) firing frequency in CA1 hippocampal neurons and decreasing the AP threshold.

The type of generated ceramides by intrinsic SMase activation in hippocampal neurons could not be determined. However, Norman's group has used mass spectrometry methods to identify the ceramides. They have elucidated that N-Smase induced increases in levels of S1P and some long chain ceramides; such as C22-Cer, C24-DHCer, glucosylceramide C16. On the contrary, levels of C20-Cer, C24-Cer, galactosylceramide C24 and gagliosides (GM1-C22) and (GM1-C24) were not affected by N-SMase. Previous studies have indicated that neuronal excitability and synaptic plasticity can be altered by the activation of receptors which are known to be coupled to N-SMase [104]. For instance, basal synaptic transmission at CA1 synapses is enhanced by the activation of TNF receptors and long term depression (LTD) at CA1 synapses is impaired with the lack of TNF receptors. The excitability of CA1 hippocampal neurons are increased by

N-SMase-mediated sphingomyelin metabolism and this metabolism causes reduced activation of the Ca^{2+}-sensitive K^+ channels which mediate membrane hyperpolarization following an AP [103]. Collectively, these findings suggest the importance of N-SMase and S1P in hippocampal neuron excitability and plasticity activities such as learning and memory.

5. CONCLUSION and FUTURE WORKS

In this study, we have investigated the effects of sphingolipids and their enzyme on *in vitro* rat hippocampal slices by using patch-clamp technique. We have recorded EPSC from hippocampal pyramidal neurons. According to our results, C2-Cer, which is one type of short chain ceramides, caused a significant depression on NMDA currents and this was an irreversible effect. On the other hand, we have worked on C2-DHCer which is the inactive form of C2-Cer. In literature, various studies indicates that inactive form of C2-Cer does not cause any effect on cells because the lack of 4,5-trans-double bond is important for bioactivity. In contrast, recent studies have found that C2-DHCer had a slight effect on neurons, but this effect less than those of C2-Cer. In our study, we found that C2-DHCer mediated a strong reducing effect on NMDA current as C2-Cer. Also, C2-DHCer effect was not reversible, therefore we thought that the effect of C2-DHCer was persistent and also neurons, on which we have worked, were still alive. We have applied the chemicals extracellularly, also we wanted to learn whether the extracellularly N-SMase is an enzyme to produce Cer, and furthermore would it generate the same effect as exogenous Cer or produce endogenous Cer. As a result of the 50 mU/ml application of N-SMase, there was a depression of NMDA current as the C2-Cer application. N-SMase receptors are located in the inner leaflet of plasma membrane and it is possible that N-SMase can be transfered into the neuron as an exosome to activate the sphingomyelin cycle. According to paired t test results, the effect of N-SMase and C2-Cer were similar. The indication of similar effects of N-SMase and C2-Cer on plasma membrane is an important observation. Moreover we have applied the N-SMase at a concentration of 30 mU/ml, even though the sample size of the group was small, the depression as the result of this was statistically significant. We found that the depression was made by 30 mU/ml N-SMase lower than those made by 50 mU/ml N-SMase application because of the concentration difference. CA1 region of hippocampus is an important part for information transfer and NMDA receptors have very critical role in the formation of synaptic plasticity; learning and memory activities. Besides it is well known that sphingolipids are component of plasma mem-

brane and their modulatory roles are very important for neuronal activity. Therefore, any disturbances caused by TNF, chemotherapeutic agents, heat stress, and ischemia to membrane sphingolipid structure components can give rise to several pathological conditions.

In this study, there is a requirement for some further experiments. For example, the sample size of 30 mU/ml N-SMase application must be increased in order to understand its role. Also, different concentrations for N-SMase can be tested to see the dose response curve. On the other hand, especially low concentrations of C2-Cer and C2-DHCer should be tested to evaluate the effects of these sphingolipids on NMDA currents. Also ATP can be added to ICF solution to study the broad effect of SMase; thus not only the plasma membrane receptors but intracellular compartment contribution as well. Moreover, some imaging techniques can be integrated to the system to observe the effects of chemicals at ionic level. Such an elaborate extention of research will provide a more detailed information about pathological mechanisms related with sphingomyeline and SMase.

APPENDIX A. CANCER and CERAMIDE

Cellular stress is a well known condition to increase ceramide levels in cells. Etoposide, vincristine, daunorubicin, doxorubicin, fludarabine, paclitaxel, PSC 833, fenretinide, and irinotecan are some cancer chemotherapeutic agents. Stimulation of the ceramide *de novo* synthetic pathway, increasing in SMase activity, or a disruption of ceramide catabolism lead to chemotherapy-mediated increases in tumor cell ceramide.

Differences in ceramide levels may contribute the cancer therapies [105]. For example, exogenous ceramide enhances paclitaxel-mediated apoptotic death of head and neck cancer cells *in vitro*. Some ceramidase inhibitors make the same effects on cancer cells as ceramide does. B13 is a ceramidase inhibitor and it increases ceramide level in tumor cells and induces tumor cell apoptosis. B13 is more active than short-chain ceramides or D-MAPP which is a ceramidase inhibitor [106]. On the other hand, ceramide-mediated death is increased by blokage of S1P biosynthesis [105]. Dimethyl-sphingosine and safingol, which is a sphingosine kinase (SK) inhibitor, decrease the formation of S1P from ceramide. As a result, pharmacological inhibition of ceramide catabolism may activate the ceramide-increasing, anticancer therapies. Furthermore, blocking the ceramide generation in normal tissues protect them from side effects of cancer therapy [105].

Some type of tumors are drug-resistant and glucosylceramide level is increased in these tumor cells. Therefore, it is suggested that the cytotoxic effects of ceramide stimulated by chemotherapy may be decreased the glycosylated form of ceramide [105]. Overexpression of glucosylceramide synthase (GCS) increases the resistance of breast cancer to doxorubicin which is a chemotherapeutic agent. Thus, tumor cells can improve their viability against some chemotherapy by glycosylation of ceramide via GCS. Moreover, cells which develop the multidrug-resistance feature are sensitive to Cer-enhancing drugs (for instance, Cer glucosylation inhibitors) caused the induction of apoptosis [107]. Additionally, Cer-enhancing drugs with anti-androgens are more effec-

tive on tumors in tissues that are androgen-dependent. GlcCer synthesis is stimulated by testosterone which inhibits GlcCer glucosidase in murine kidney [107]. Therefore, poly-drug therapy is useful for renal cancer and also it is possible that this approach can be useful for prostate and lung cancer [107]. Stimulation of Cer synthase in prostate cancer with phorbol diester enhances the response to radiation so the level of Cer is increased and early apoptosis is induced [108].

γ-irradiation and cytotoxic drugs; such as doxorubicin activate the CD95 system of apoptosis [109]. Furthermore, the level of ceramide is increased by these inducers. Herr's group applied C2-Cer on leukemic T cells for 16 h and the application induced apoptosis [109]. In addition, 3-4 h after the application, CD95-L protein expression is increased. Moreover, CD95-L expression and apoptosis are specific for C2-Cer. It is biologically active form of C2-DHCer which is inactive in tumor cells.

Additionally, the level of ceramide is decreased in human colon cancer when compared with normal colon cells. [106]. Selzner et al. indicated that there were three major ways that ceramide activated proapoptotic mechanisms: (1) ceramide activated caspases indirectly and this caused DNA fragmentation; (2) ceramide activated the stress-activated protein kinases (SAPK/JNK) pathway. This activation induces apoptosis by a caspase 3-dependent mechanism; (3) ceramide regulated TNF receptor 1 and Fas receptors on the cell membrane [106].

In conclusion, the roles of ceramide in cancer therapies need to be more well defined. Therefore, further investigations are required to clarify the effects of different ceramides on different types of tumor cells. Also, it is important to generate the poly-drug mixtures that can elevate Cer levels and lower the levels of anti-apoptotic sphingolipids.

APPENDIX B. BARRIER FUNCTION of SKIN and CERAMIDE

Stratum corneum (SC) which is produced by the epidermis is protective and semi-permeable barrier to permit terrestrial life. Lipid lamellae which is localized to the extracellular spaces between corneocytes provides the barrier function of SC. SC is composed of ceramides, cholesterol, and free fatty acids but lacks phospholipids [110, 111]. 45-50% of lipid weight is ceramide, 25% of the weight is cholesterol, and 10-15% of the lipid composition is free fatty acids. In 1980s, the detailed structure of the ceramide species of human skin were determined and it is still being refined. In Figure A1, human SC ceramide structures are shown [112].
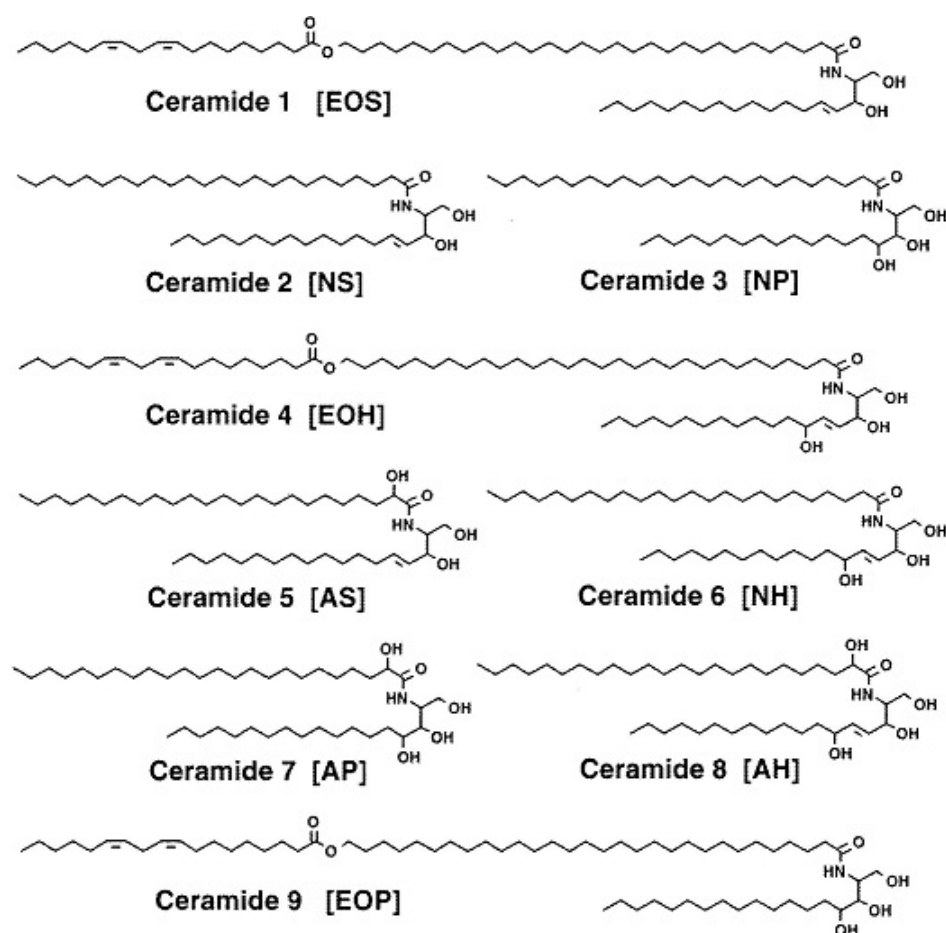
Figure B.1 Structures of the ceramide in human SC. Numbers (1-8) represent thin layer chromatographic mobility. Ceramide 1 is the least polar and ceramide 8 is the most polar ones. Ceramide 9 has a thin layer chromatographic mobility [107].

C26-C32 ceramides are long chain, saturated fatty acids and they are the principal stratum corneum lipids. Moreover, in marine mammals, the short chain and N-acyl species (shorter than C16) substitute the long chain and saturated species, because marine mammals need less tight barrier [110]. Both cholesterol and fatty acid synthesis are important for permeability barrier homeostasis. This has been proved by using some solvents, which remove sphingolipids from the SC, abrogate the permeability barrier [113].

Even though the effect of psychological or exertional stress on human skin has not been well studied, in the last decade some effects on barrier function of skin has been identified. Altemus's group has worked on three different stressors [114]. One of the stressors was pscyhologic interview stress which caused a delay in the recovery of skin barrier function. Moreover, the interview stress increased the norepinephrine, plasma cortisol, interleukin-1β and interleukin-10 (IL-10), tumor necrosis factor-α, and circulating natural killer cell activity and natural killer number. The other stressor was sleep deprivation used by Altemus et al. [114]. They have found that sleep deprivation decreased the recovery of skin barrier function but increased plasma interleukin-1β, TNF-α, and natural killer cell activity. Also exercise stress was used as the last stressor by Altemus's group [114]. They have reported that the exercise stress did not affect the recovery of skin barrier function but caused an increase in natural killer cell activity and circulating numbers of both cytolytic T lymphocytes and helper T cells. According to them, there was a inverse correlation between cytokine responses and changes in barrier function recovery. They have suggested that the homeostasis of skin barrier function in women was disrupted by acute psychosocial and sleep deprivation, and this disruption may have been related to stress-induced changes in cytokine secretion. Furthermore, disruption of barrier function increased epidermal cell proliferation and DNA synthesis [115], synthesis of keratins and secretion of TNF-α, IL-1α, IL-1β, IL-8, IL-10, TGF-α (transforming growth factor), and TGF-β but not TNF-β, IL-2, IL-3, IL-4, or IL-5 [116]. The TNF signaling pathway facilitates epidermal barrier function recovery via activation of sphingomyelinase [114]. Although IL-1 activates the sphingomyelinase pathway, TNF-α plays a more important role in barrier function repair.

On the other hand, ceramide level is important for atopic dermatitis and psoriasis which are both inflammatory skin diseases [112]. In atopic dermatitis, the reduction in the amount of ceramides is the most consistent finding [117]. Also TNF-α has been reported in a wide range of inflammatory conditions, as atopic dermatitis [114]. In psoriasis, the abnormal lipid structure and alterations in ceramide content have been demonstrated [112].

The barrier function of skin prevents the transepidermal water loss (TEWL) [112]. The term moisturizer means that the material applied adds water and/or retains water in the SC. The mechanism of preventing TEWL may vary, however these materials have different unknown or less well defined effects on SC functions. Urea, propylene glycol, glycerin, and hydroxy acids which are humectants are mostly used in moisturizing formulations but they promote desquamation [112]. An exogenous barrier to TEWL is another mechanism of moisturizers to retain water in the SC. This barrier creams form a film on the skin surface. Nevertheless, the contribution of certain lipid combinations to restoration of barrier function is still not well defined.

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