INVESTIGATING LIPID SIGNALING IN RAT HIPPOCAMPAL SLICES WITH PATCH-CLAMP TIGHT SEAL WHOLE CELL RECORDING TECHNIQUE

by

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ABSTRACT

INVESTIGATING LIPID SIGNALING IN RAT HIPPOCAMPAL SLICES WITH PATCH-CLAMP TIGHT SEAL WHOLE CELL RECORDING TECHNIQUE

Ceramide, besides its structural role in cell membrane as a sphingolipid, has essential roles in apoptosis, cell growth and differentiation. In this study, the effect of C2-ceramide (10 μM) application on whole cell currents recorded by patch-clamp technique from cell body of hippocampal CA1 pyramidal neurons was investigated. Evoked post-synaptic currents were recorded with low frequency (0.1 Hz) stimulation of Schaffer collateral. It was observed that ceramide application resulted with depression among the NMDA currents. On the other hand, there was not any significant influence on non-NMDA currents. The role of C2-ceramide which leads to the depression on the NMDA currents showed that sphingolipids have an effect directly on the cell membrane, besides their roles as secondary messengers inside the cell.

Keywords: C2-ceramide, hippocampal synaptic currents, NMDA and non-NMDA currents, patch-clamp tight seal whole cell technique

ÖZET

SIÇAN HİPOKAMPAL KESİTLERİNDE LİPİT SİNYAL İLETİMİNİN TEK HÜCREDE SIKI BAĞLI YAMA-KENETLEME KAYIT TEKNİĞİ KULLANILARAK ARAŞTIRILMASI

Hücre zarındaki bir sfingolipit molükülü olan seramit, hücre zarının bir yapı elemanı olma özelliğinin yanısıra, apoptoz, hücre büyümesi ve hücre farklılaşması olaylarında önemli rol oynamaktadır. Bu çalışmada, C2-seramitin (10 μM) hipokampal CA1 piramidal hücre gövdelerinden patch-clamp tekniğiyle kaydedilen tüm hücre akımları üzerindeki etkisi araştırılmıştır. Schaffer kollateral, düşük frekansta (0.1 Hz) uyarılarak oluşan postsinaptik akımlar kaydedilmiştir. Seramitin NMDA akımı üzerinde baskılayıcı bir etkisi olduğu gözlenmiştir. Buna karşın NMDA olmayan akımlar üzerinde belirgin bir etkisi gözlenmemiştir. Seramitin NMDA akımı üzerindeki bu seçici etkisi, sfingolipitlerin hücre içi kimyasallar yoluyla oluşturduğu etki mekanizmasının yanısıra, doğrudan hücre zarı üzerinde de bir etkisi bulunduğunu göstermektedir.

Anahtar Sözcükler: C2-seramit, hipokampal sinaptik akımlar, NMDA ve NMDA olmayan akımlar, tek hücrede sıkı bağlı yama-kenetleme tekniği

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LIST OF ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate
AP5	2-amino-5-phosphonopentanoic acid
A-SMase	Acidic sphingomyelinase
CA	Cornu ammonis
CNS	Central nervous system
CPP	3-[(±)-2-carboxypiperazin-4-yl] propyl-1-phosphonic acid
DMSO	Dimethylsulfoxide
DNQX	7-Dinitroquinoxaline-2,3-dione
EPSC	Excitatory post-synaptic current
FB1	Fumonisin B1
GluR	Ionotropic glutamate receptor
LTP	Long term potentiation
NMDA	N-methyl-D-aspartate
N-SMase	Neutral sphingomyelinase
p75NTR	75-kDa neurotrophin receptor
SM	Sphingomyelin
SPT	Serine palmitoyltransferase
TNFα	Tumor necrosis factor-alpha

1. INTRODUCTION AND MOTIVATION

In the last decades, neuroscience studies advanced by integrating several research lines and approaches in this field. Improvements in the experimental techniques helped researchers in obtaining a more detailed understanding of the brain and the nervous system. Outcomes of the clinical studies has manifested significant amount of questions to be answered. From computational studies to experimental approaches there are so many disciplines which are currently dealing with the hot topics of the neuroscience all over the world.

Now, clinical studies and basic scientific studies are in close cooperation in their research body. Most of the neurological disorders in the central nervous system, although identified more than hundred years ago, has not been very well understood. By the advance in experimental techniques in neurobiology, physiology and related fields, it comes closer to get more detailed understanding on the basis of the disorders and as a result, better therapatic applications.

One of the common neuronal disorder is Alzheimer's disease. It has long been investigated however molecular basis of the disease has not yet been fully understood. The major clinical observation of the disease is the neuron death in the hippocampal region of the brain. This brain region can be visualized by imaging techniques like magnetic resonance hence neuron degeneration is visible. Increasing attention in the mechanism of neuronal death has accelerated the molecular studies and *in vitro* approaches in this field. One of the major outcome of the neuron degeneration is the disturbance or loss of the excitability patterns in neurons, which is a critical parameter in the transmission of information in the nervous system.

Investigating the excitability of neurons is a suitable plane for electrophysiological studies. By using electrophysiological techniques, it is possible to track the changes in the neuron by means of electrical currents passing through the cell to transmit an

electrical signal. Changes in the transmission of an electrical signal in the nervous system is directly related with the excitability of the neurons. Excitability of neurons can be affected with several insults from the outside. Those insults may induce changes in the receptors activity located on the cell membrane which have critical roles in synaptic transmission.

In this study, we questioned the change in the excitability of the neurons in the central nervous system, particularly in the hippocampus region of the brain in response to sphingolipid application. Although there are many studies in molecular level on the effects of sphingolipids on the neuronal activity, our electrophysiological approach by using patch-clamp tight seal whole cell recording technique, can provide new implications in determining the concrete effects of sphingolipids in terms of electrical excitability.

2. BACKGROUND

2.1 Hippocampal Formation

The hippocampal formation of the mammalian brain has long been attracted the neuroscientists, psychologists, physiologists and neuroanatomists. The hippocampal formation consists of a number of subdivisions: hippocampus proper, dentate gyrus (DG), entorhinal cortex (EC) and subiculum (fig. 2.1) [1] .

The hippocampus, (hippo = horse, kampos = sea monster; in Greek; similarity of its shape with sea horse) residing in the hippocampal formation, is among the best characterized cortical structures. First drawings of Cajal show the stratified pattern of hippocampus (fig. 2.2). In this stratified body, the cell bodies are gathered in a single layer and relatively precise organization of the afferents are making synapses on the dendrites. This structural presentation of hippocampus is a good model for electrophysiological studies of neuronal excitability.

Hippocampus is part of the limbic system. In this system, hippocampus has an essential role in certain aspects of learning and memory [2]. In this processes, hippocampus has a central role and also structurally takes position at a critical place in the brain. The hippocampus is adjacent and functionally connected to the perirhinal, parahippocampal and entorhinal cortices [3]. In addition, hippocampus is the contact point between the temporal cortex and the frontal cortex, which is involved in different types of mental tasks [4].

In the formation of declarative memory, which is also called as explicit and relational memory, hippocampus (together with anatomically related structures) plays a key role. The hippocampus is necessary to bind the information distributed sites in neocortex those together represent a whole memory [7]. The information stored in the hippocampus remains vulnerable to interferences until it is transferred into other brain

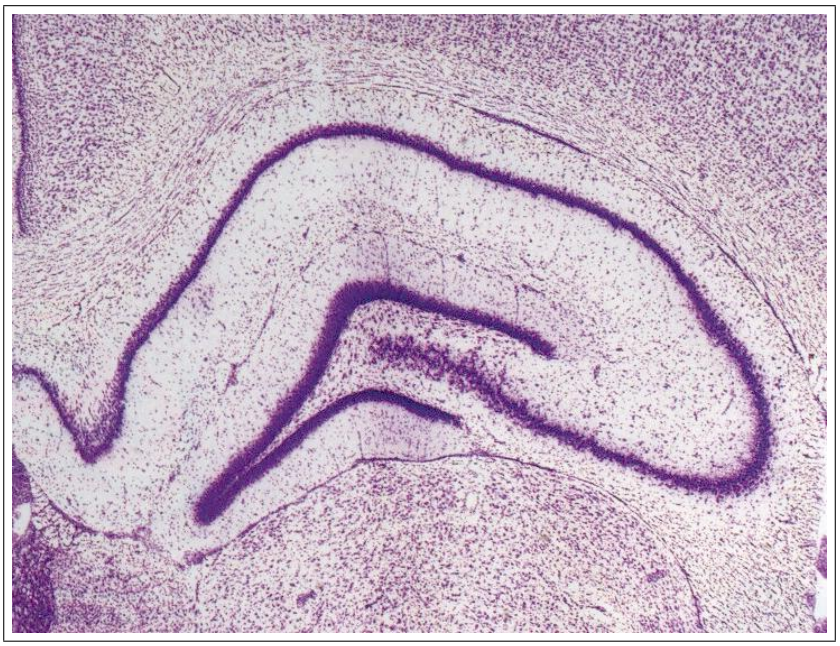

Figure 2.1 Coronal section of the hippocampus, stained with cresyl violet [5].

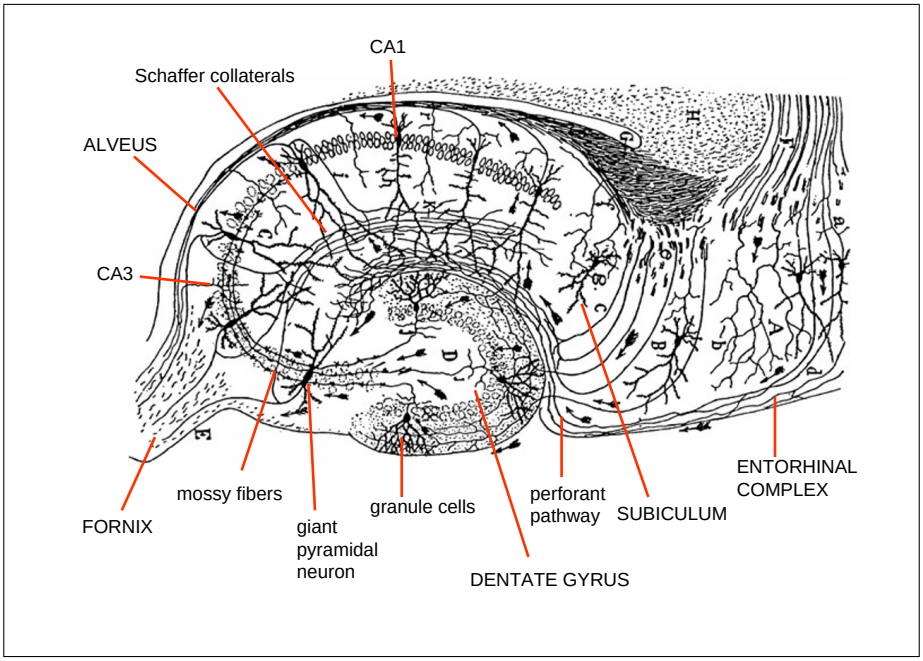

Figure 2.2 Hippocampal formation of the rodent hippocampus. The hippocampus and nearby regions in the temporal lobe, the dentate gyrus and the subiculum, collectively form the hippocampal formation. (Adapted from Cajal, 1911 [6]).

structure (such as the cortical regions) where memories are fixed and seem to be less vulnerable to interference.

The hippocampus, as well as learning and memory processes that depend on proper hippocampal function, is particularly vulnerable to aging [8]. Aging of animals and humans have shown an impairment in hippocampus-dependent learning tasks [9]. Pathophysiology of hippocampus can have serious clinical consequences and this is also another reason for studying hippocampus. As an example, it is a target of neurodegenerative disorders like Alzheimer's disease. In addition, this structure is prone to seizure and is commonly involved in temporal lobe epilepsy [10].

There are several research lines to understand the cellular mechanism underlying normal and abnormal forms of synchronization of hippocampal neuronal activity. By the progress in intracellular recordings of hippocampal neurons, scientific studies are advanced in detailed understanding of the cellular neurophysiology of the hippocampus.

2.2 Excitatory Pathways in Hippocampus

The transverse lamellar organization permits the use of hippocampal slices with preserved circuitry where stable, long-lasting and high-quality intracellular recordings can be performed [11]. In this circuitry, two interconnected crescent-like regions are clearly visible: Ammon's horn (cornu ammonis, CA) and dentate gyrus.

Ammon's horn is a curved structure, it has a laminar organization. On a coronal section, Ammon's horn of the rat can be subdivided into two regions: CA1 and CA3; in humans, two other subdivisions exist, CA2 and CA4. While CA2 lies between CA1 and CA3, CA4 lies in dentate gyrus. The principal cells, pyramidal cells, have their soma aligned in a thin layer called the pyramidal cell layer. Those cells have dendritic tree that resembles a pyramid shape, so they are called pyramidal neurons. Three main dendritic trunks emerge from the cell body, one apical and two basal. Apical dendrites extend in the stratum radiatum and arborize in stratum lacunosum

moleculare. Basal dendrites of the pyramidal cells form stratum oriens. Axons of pyramidal cells go through stratum alveus where they emit numerous collaterals before leaving the hippocampus.

The dentate gyrus is also a curved structure, and formed from three layers: molecular layer, granule cell layer and polymorphic cell layer (hilus). The principal cells, granule cells, form a densely packed thin layer, named as the granular cell layer. Dendritic trees emerge from the apical pole of somas and form the molecular layer. Axons of the granule cells form mossy fibers. They have a relatively small diameter and they are not myelinated. They emerge from the basal pole of somas and form polymorphic cell layer and divide into numerous collaterals. Those collaterals contact local interneurons, and cross the hilus. Afterwards they make synapses with CA3 pyramidal cells.

Hippocampus receives afferent input from two major pathways: perforant pathway coming from entorhinal cortex and the fornix coming from the medial septum and anterior thalamus. There are also important but less numerous projections from several other regions including brain stem, hypothalamus, thalamus and amygdala (fig. 2.3). The entorhinal cortex provides a major sensory input to the hippocampus via the fibers of the alvear and perforant pathways. The entorhinal cortex receives input from many other regions of the brain like association cortices, the olfactory cortex, several thalamic nuclei, the claustrum and the amygdala. The perforant pathway originates in layer II and III of the entorhinal cortex and perforates the hippocampal fissure before terminating in all parts of the hippocampus [13]. Second significant input originates in the medial septal nucleus and the nucleus of the diagonal band. It enters the hippocampus by four routes: the fimbria, the dorsal fornix, the supracallosal striae and the amygdaloid complex. The termination sites are distributed throughout the hippocampus, but preferentially in region CA3 and the dentate gyrus. Commissural fibers from the contralateral hippocampus are also another type of input. These fibers enter the fimbria and then collect in the fornix. The two fornices are connected dorsally by the hippocampal commissure.

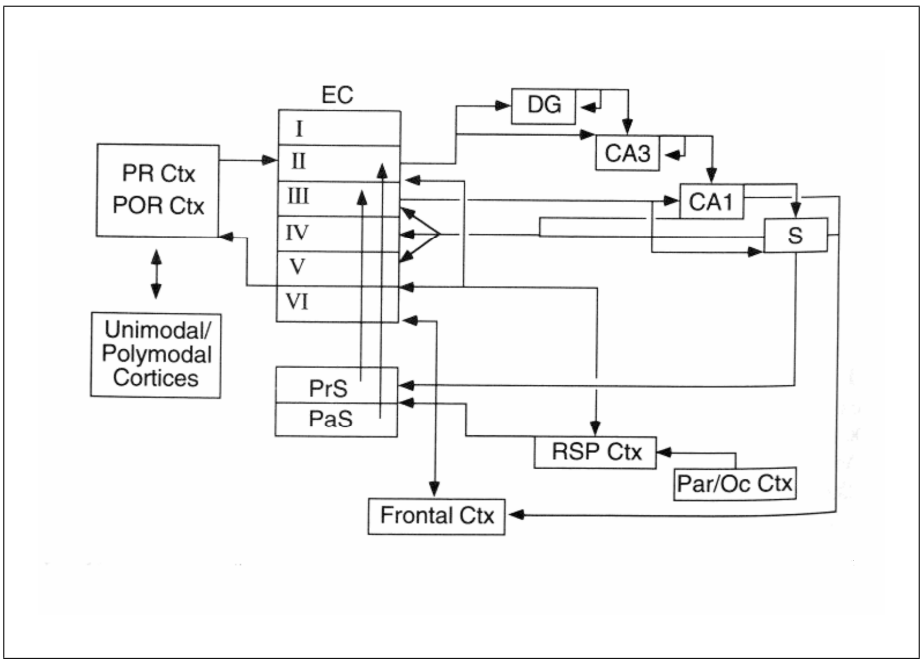

Figure 2.3 Intrinsic connections and extrinsic inputs of the hippocampal formation. The diagram emphasizes the serial and parallel aspects of the intrinsic hippocampal circuitry. DG, dentate gyrus; EC, entorhinal cortex; PR, perirhinal; POR, postrhinal; PrS, presubiculum; PaS, parasubiculum; Par/Oc Ctx, parietal occipital cortices; RSP Ctx, retrospinal complex (adapted from Johnston et.al. 1998) [12].

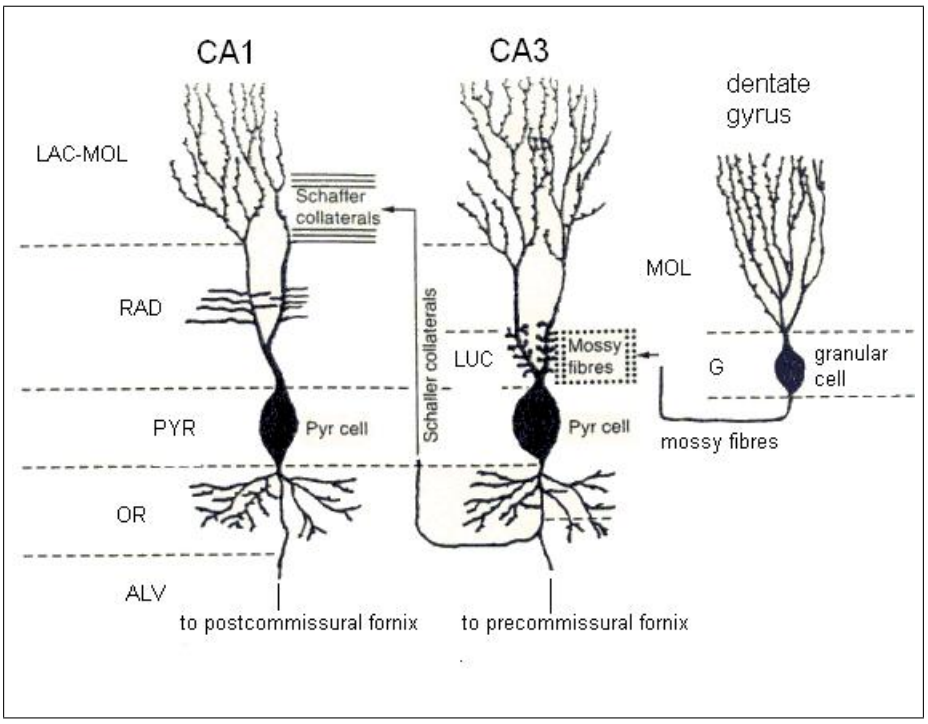

Figure 2.4 Trisynaptic circuit is composed pyramidal neurons placed in three different location: CA1, CA3 and dentate gyrus. Hippocampus is divided mainly into 4 main layers: stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR) and stratum lacunosum-moleculare (SL). Axons of granular cells form mossy fibers. Axonal collaterals of CA3 pyramidal cells are called Schaffer collaterals (adapted from Hammond, 2001) [14].

Principal cells of the hippocampus establish the main circuit (tri-synaptic circuit) of the hippocampus (fig. 2.4). Tri-synaptic circuitry is formed between granular cells of dentate gyrus, pyramidal cells of CA1 and pyramidal cells of CA3. Circuitry begins with granular cells which project on to CA3 pyramidal cells and form synapses. Mossy fibers of the granule cells, terminate on the proximal portion of CA3 apical dendrites, on to giant spines, known as thorny excrescences. This restricted zone of projection forms the stratum lucidum, a sublayer of the radiatum that exists only in the CA3 region. Synapses between mossy fibres and dendritic spines of CA3 pyramidal cells are giant synapses. The pyramidal neurons of the CA3 region send axons out of the hippocampus in the fornix. A branch of this axon forms Schaffer collateral and innervates the CA1 pyramidal cells, on the distal part of their apical dendrites at the level of stratum lacunosum moleculare. Schaffer collateral synapses are among the most extensively studied in the mammalian brain, particularly for *in vitro* electrophysiological experiments [15]. Interneurons located in the hippocampus are innervated by the axons of both granular cells and pyramidal cells. Pyramidal cells of the CA3 and CA1 regions emit local axon collaterals those contact local interneurons. Similarly, granular cells emit axon collaterals those locally innervate interneurons. Moreover, CA3 pyramidal cells are connected to each other by excitatory recurrent collaterals.

2.3 Ionotropic Glutamate Receptors in the Hippocampus

Glutamate is an important neurotransmitter of cortical and hippocampal pyramidal neurons acting on glutamate receptors. Glutamate receptors consist ligand gated ion channels (called as ionotropic receptors) and G-protein-coupled metabotropic receptors. Ionotropic glutamate receptors are classified according to structural homology, kinetics and relative binding affinity for the pharmacologic agonists.

The presynaptic release of glutamate activates two types of postsynaptic glutamate receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors. NMDA, AMPA and kainate receptors are protein complexes located in the membrane and composed of several subunits. There

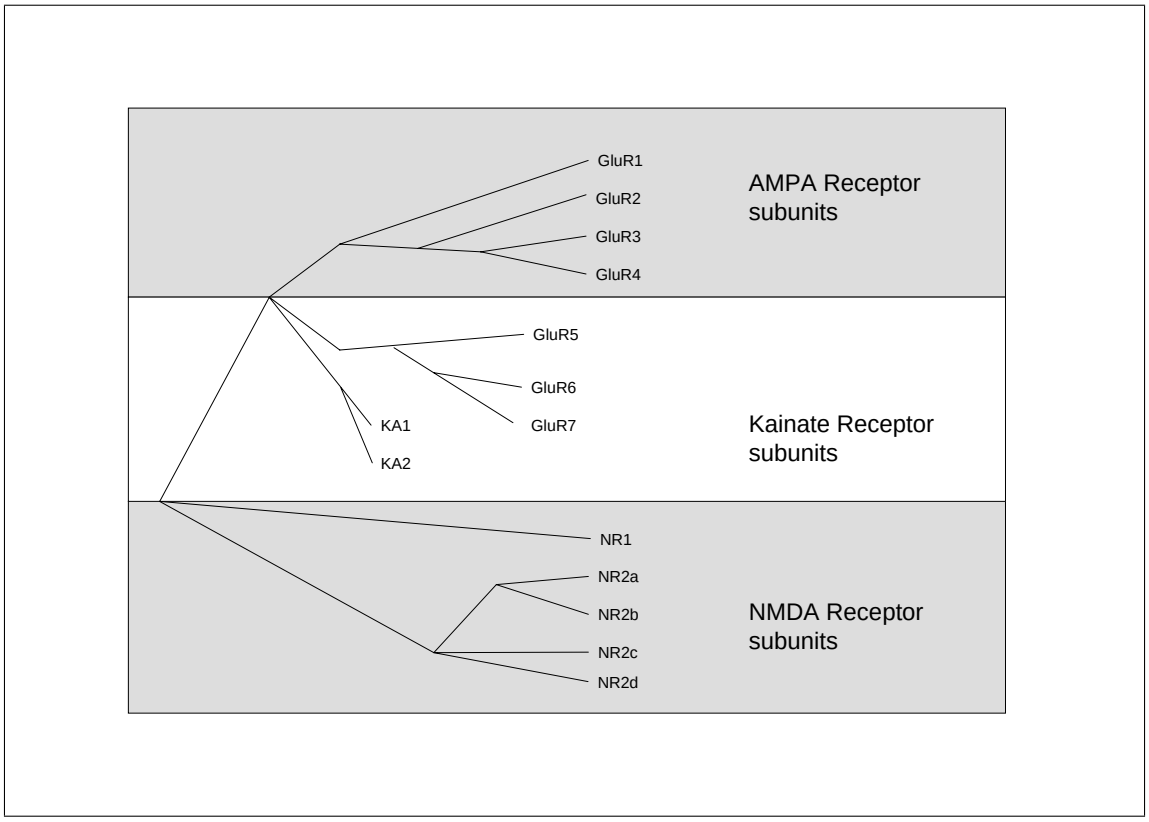

Figure 2.5 Subunits of ionotropic glutamate receptors. The ionotropic glutamate receptors are multimeric assemblies of four or five subunits, and are subdivided into three groups (AMPA, NMDA and Kainate receptors) [14].

are 14 cDNAs identified, four for AMPA receptor subunits (GluR1, GluR2, GluR3, GluR4), five for kainate receptor subunits (GluR5, GluR6, GluR7, KA1 and KA2) and five for NMDA receptor subunits (NR1, NR2A, NR2B, NR2C and NR2D) (Fig 2.5). Ionotropic receptor (iGluR) is composed of a large extracellular N-terminus domain, four hydrophobic segments (TMI-IV) and intracellularly located C-terminus. The TMII segment forms a re-entrant loop. In addition, the long loop between TMIII and TMIV is exposed to the cell surface and forms part of the binding domain with the C-terminal half of the N-terminus (Fig 2.6).

2.3.1 NMDA Receptor

The NMDA receptor is an ionotropic receptor. Its ligand, glutamate, binds its agonist site for the activation of the receptor. Receptor name comes from NMDA (N-methyl D-aspartate) which is receptor's selective specific agonist. As a result of receptor activation, Na^+ and small amounts of Ca^{2+} ions flow into the cell and K^+ ions flow out of the cell.

Structurally, NMDA receptors are heteromeric integral membrane proteins and composed of NR1 and NR2 subunits. Each receptor subunit behaves as a modular structure and each structural module represents a functional unit. NR1 subunits bind the co-agonist glycine and NR2 subunits bind the neurotransmitter glutamate (Fig 2.7). Agonist binding domain links to membrane domain which is carrying transmembrane segments and a re-entrant loop. The membrane domain is also a part of the channel pore and is responsible for the receptor's conductance and voltage-dependent magnesium block. Beside these, each subunit has an extensive cytoplasmic domain. These cytoplasmic domains are affected by protein kinases and protein phosphatases. Functional modulation of the receptor can be achieved through actions at various recognition sites including the primary transmitter sites. Competitive type of modulation on glutamate binding site is carried out by 2-amino-5-phosphonopentanoic acid (AP5), 3-[(±)-2-carboxypiperazin-4-yl] propyl-1-phosphonic acid (CPP) [16]. Non-competitive type of modulation, behaving as channel blocking is carried out by MK-801,

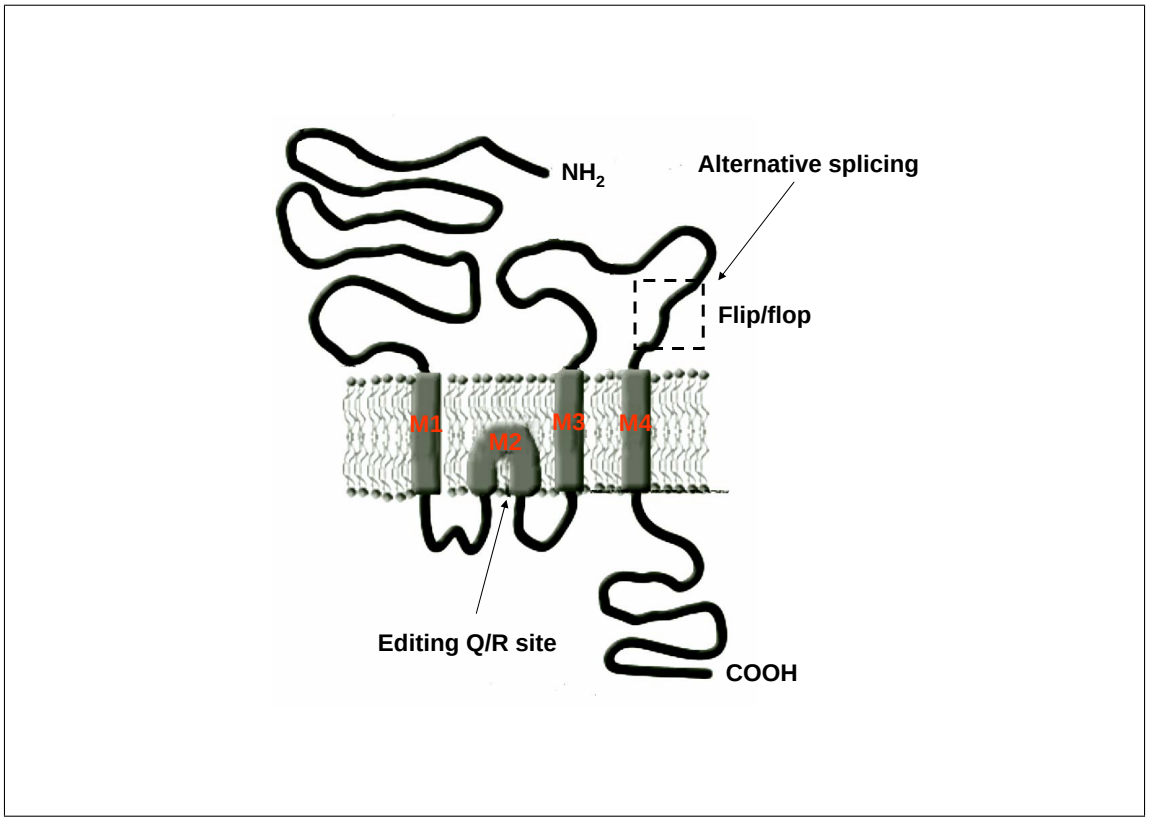

Figure 2.6 Structure of glutamate receptor. All ionic glutamate receptors have a similar structural pattern: an extracellular N-terminus and a large loop for the ligand binding domain; an intracellular C-terminus where is the site of splice variation and the site of interaction with intracellular proteins.

ketamine, memantin, PCP, Mg^{2+}. The NR2B subunit also posesses a binding site for polyamines, regulatory molecules that modulate the functioning of the NMDA receptor. Polyamines, via an intracellular site, affect the NMDA receptor-channel complex, non-competitively. Co-agonist glycine is necessary for the receptor activation via its binding site [17]. Kynuronic acid act as an antagonist, blocks glycine binding site and inhibits receptor activity [18].

At resting membrane potential, NMDA receptors are inactive. This is due to a voltage-dependent block of the channel pore by magnesium ions, preventing ion flux. Sustained activation of AMPA receptors by a train of impulses arriving at a pre-synaptic terminal, depolarises the post-synaptic cell, releasing the channel inhibition and allowing NMDA receptor activation [19–21]. Activation of NMDA receptors results with Ca^{2+} influx in addition to the Na^{+} influx and K^{+} efflux. Such calcium influx has been implicated in a range of physiologic phenomena including long-term potentiation, (LTP) synaptic integration and plasticity. Calcium influx may induce various signaling pathways that can act locally to produce immediate changes in the synapse or under repeated stimulation, can affect gene expression responsible for long-lasting LTP. On the other hand, high levels of glutamate as a result of NMDA receptor overexcitation, may lead to neurotoxicity. Such excess glutamate in the extracellular space (seen in Alzheimer's disease (AD)) [22], produces mitochondrial dysfunctions, energy deprivation and a persistent partial cell depolarisation. All those cascades of events lead to NMDA receptors activation by unblocking of Mg^{2+} and result with an undesired increase of intracellular Ca^{2+}. Calcium accumulation inside the cell triggers several signal cascades responsible for oxidative stress and neuronal death [23]. Beside the calcium influx through the channel, alterations in the transmission of neuronal information via NMDA receptors may also arise due to the unique profile of the receptor-channel complex, which requires various conditions for operation, and therefore is not necessarily involved in synaptic transmission at all times and under all circumstances.

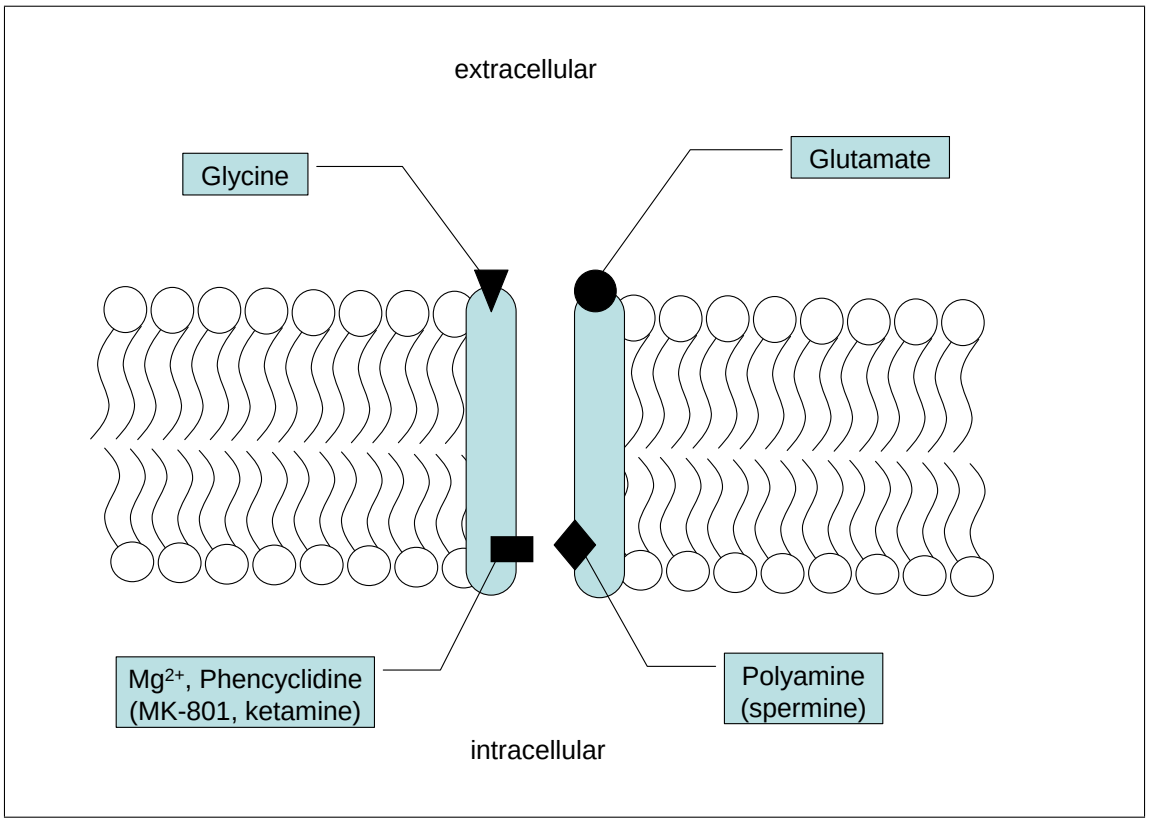

Figure 2.7 There are distinct binding sites for the modulation of NMDA receptor. The channel associated with the receptor is blocked by Mg^{2+} at resting potential (-70 mV). Removal of Mg^{2+} block is necessary for receptor activation as well as the binding of glutamate and the co-agonist, glycine (ligand-gated). The different binding sites (glutamate, phencyclidine, polyamine, glycine) are shown, and together with antagonists at various sites. The polyamine site is an intracellular site which modulates the affinity of other agonists and antagonists.

2.3.2 Non-NMDA Receptors (AMPA and Kainate Receptors)

AMPA receptors are involved in the fast synaptic transmission in the central nervous system. AMPA receptors are composed of 4 subunits, GluR1-4. The ligand binding domain is made up from N-terminal regions S1 and S2 while the C-terminus contains binding sites for several proteins. In excitatory neurons, the initial stage in excitatory synaptic transmission is a fast-depolarising response due to the release of glutamate, subsequent activation of the AMPA receptor and an immediate sodium influx into the cell. Native AMPA receptor channels are impermeable to calcium, a function controlled by the GluR2 subunit. The calcium permeability of the GluR2 subunit is determined by the post-transcriptional editing of the GluR2 mRNA. As a result, a single amino-acid in the TMII region changes from glutamine (Q) to arginine (R). This is the so called Q/R editing site. GluR2(Q) is calcium permeable while GluR2(R) is not. In CNS, almost all the GluR2 protein expressed is in the GluR2(R) form, giving rise to calcium impermeable AMPA receptors.

Although kainate receptors share many of the same structural characteristics with AMPA and NMDA receptors, they constitute a separate group from the NMDA and AMPA receptors. They are built from multimeric assemblies of GluR5-7 and KA-1/2 subunits. They possess an extracellular N-terminus that, together with a loop between TMIII and TMIV, forms the ligand binding domain and a re-entrant loop (TMII) that forms the lining of the pore region of the ion channel. They undergo splice variation and RNA editing which leads to a large number of possible receptors with differing pharmacological and functional properties. The ion channel is permeable to sodium and potassium ions. The amount of sodium and potassium that the channels allow through their pores per second is similar to that of AMPA channels. However, the openings of kainate receptors are much shorter in duration than AMPA openings. Their permeability to Ca^{2+} is usually very short but varies with subunits and RNA editing [24].

2.4 Cell Membrane and Sphingolipids

2.4.1 Cell Membrane

Biologic membranes are not simply inert physical barriers, but complex and dynamic environments those affect membrane protein structure and function. Biophysical studies advanced after the interpretation of the fluid mosaic model by Singer and Nicolson [25]. In this model, structure of the plasma membrane was explained as a homogeneous phospholipid bilayer in which integral membrane proteins reside. In the last decade, by the impact of new concepts in cell membrane like lateral heterogeneity, lipid microdomains, and rafts, a more heterogenous dinamic entity was introduced. In that concept, there exist microdomains, which are enriched with many kinds of lipids such as cholesterol, glycolipids, and sphingolipids, present in cell membranes [26].

2.4.2 Structure of Sphingolipids and Their Functions

Lipids are dynamic structures in the cell membrane. Sphingolipids are a group of lipid molecules and an essential part of the cell membrane (fig. 2.8). Sphingolipids were firstly found in the brain and its backbone was named as sphingosine resembling the sphinx in Greek mythology due to its enigmatic properties [27]. A sphingolipid moiety is composed of one molecule of the long-chain amino alcohol sphingosine or one of its derivative, one molecule of a long-chain acid, a polar head alcohol and sometimes phosphoric acid in diester linkage at the polar head group (fig. 2.9).

2.4.3 Sphingolipids and Cell Metabolism

There are currently over 300 known sphingolipids with distinct headgroups. All contain a base (sphingoid) backbone. In mammalian cells, the most common sphingoid base is D-erythro-sphingosine. More complex sphingolipids can be synthesized including the major sphingophospholipid, sphingomyelin (SM), by addition of phos-

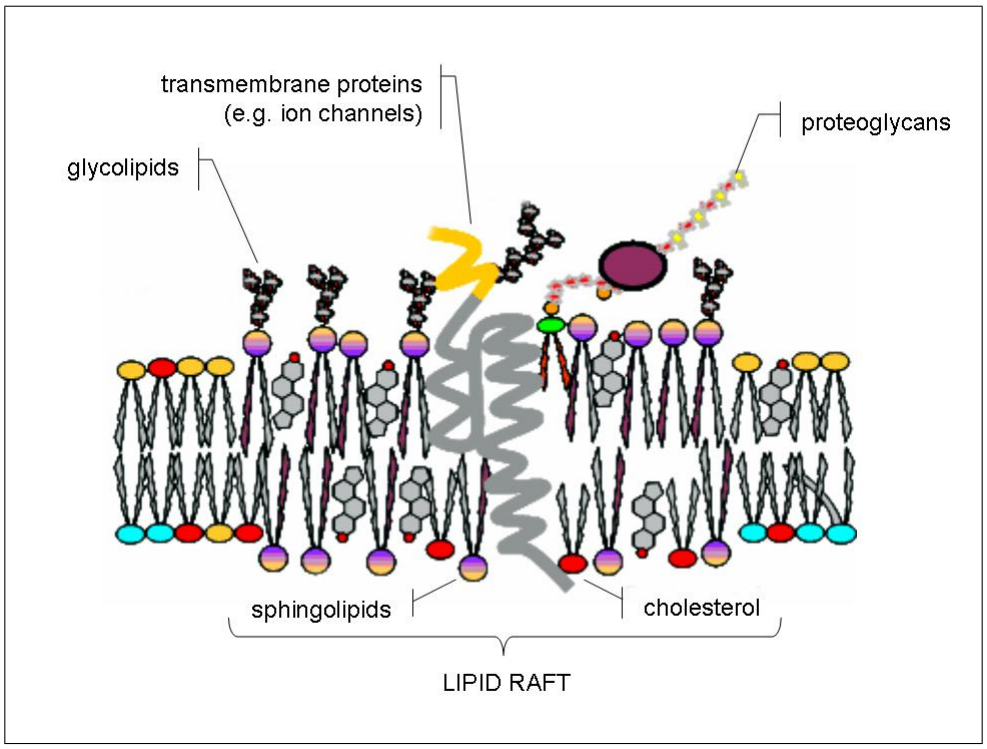

Figure 2.8 Structure and organization of a lipid raft microdomain in the plasma membrane. Sphingolipids, which include both sphingomyelin and glycosphingolipids, associate with cholesterol to form a more tightly packed domain. The regions rich in phosphatidylcholine and other glycerol-based phospholipids are less densely packed, and form fluid regions outside the raft microdomains.

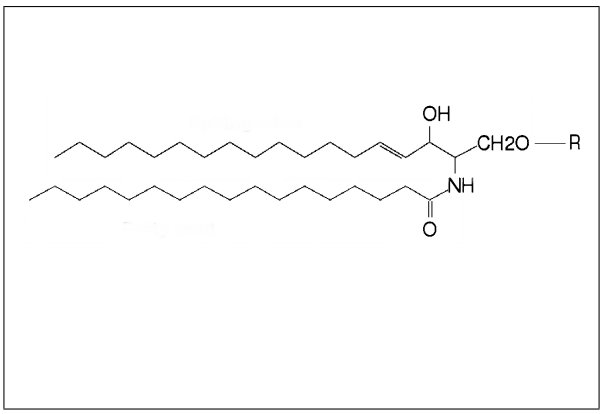

Figure 2.9 The sphingosine backbone is O-linked to a (usually) charged head group such as ethanolamine, serine or choline. The backbone is also amide-linked to an acyl group, such as a fatty acid.

phocholine. Glycosphingolipids can also be synthesized by addition of glucose to the central sphingolipid molecule [28].

Sphingolipids have been long regarded as structural and inert components of cell membranes [29]. In last two decades, besides their physical roles in cell membrane, it was observed that sphingolipids are involved in intracellular activities. Studies in molecular cell biology have shown that lipid signaling molecules derived from sphingomyelin cycle have role in signal transduction in the cell, in mediating cellular processes such as growth, differentiation and apoptosis [30–36]. Over the past decade, sphingolipid metabolites including ceramide, sphingosine, and sphingosine 1-phosphate have emerged as a new class of lipid biomodulators of various cell functions. In between those three metabolites, ceramide has been shown to mediate growth arrest, differentiation, and apoptosis. Sphingosine, the N-deacylated product of ceramide, is also capable of inducing apoptosis. In contrast to ceramide and sphingosine, however, sphingosine 1-phosphate has been implicated in mediating cell proliferation and antagonizing ceramide-mediated apoptosis [29, 35, 37, 38].

2.4.4 Ceramides: A Key Sphingolipid in Cell Metabolism

Ceramides, constitute the hydrophobic backbone of all the complex sphingolipids: sphingomyelin, cerebrosides, gangliosides, and others. The fatty acid chain length of ceramide can vary from 2 to 28 carbons, although C-16 to C-24 ceramides are most abundant in mammalian cells. These fatty acids are usually saturated or monounsaturated, and sometimes may contain a hydroxyl group at the C-2 position (K-hydroxy fatty acid) or on the terminal C atom (g-hydroxy fatty acid) [39]. Naturally found ceramides contain a long-chain fatty acyl residue, and are highly hydrophobic. They do not exist free in aqueous media-in cytosol- but are found instead in the cell membranes. Consequently, the primary targets of ceramides are proteins either permanently or transiently related to membranes. Ceramides may directly bind a specific site in the protein, or modify the bilayer properties, thus influencing protein function. Thus, they can exert their physiological effects either through changes in membrane properties

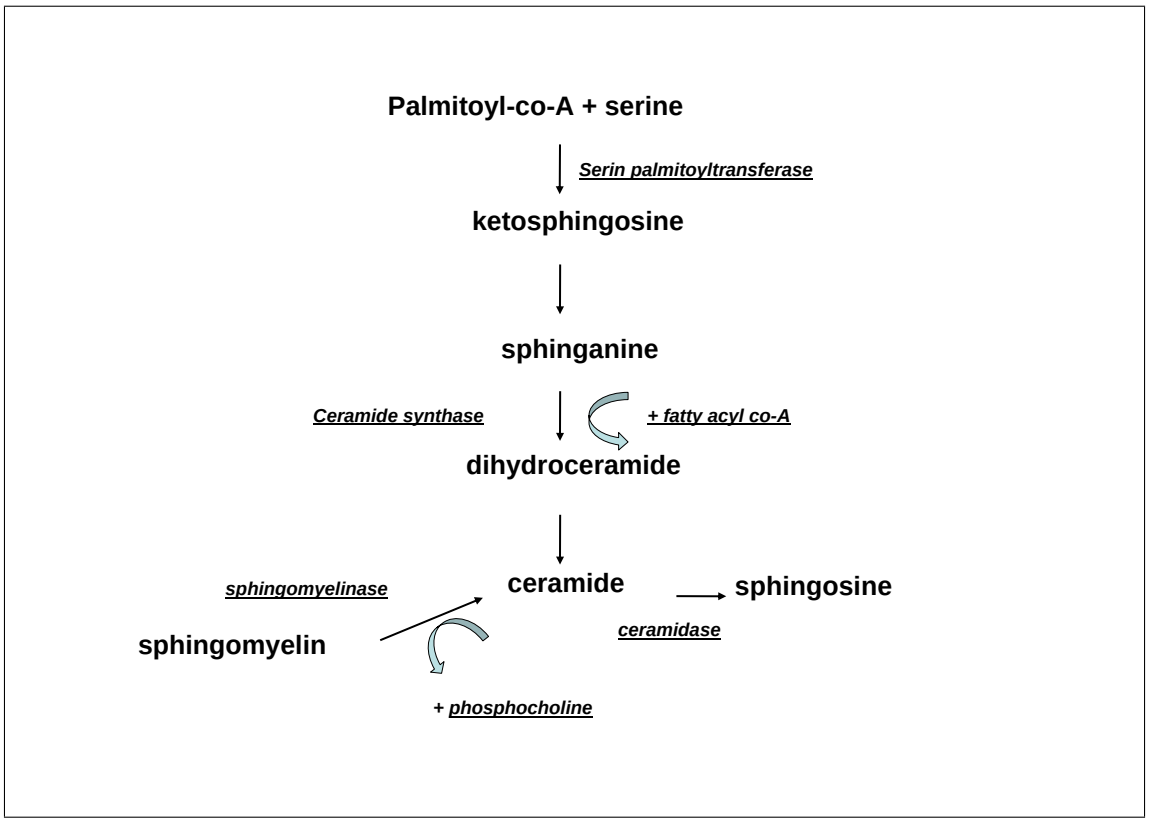

Figure 2.10 *De novo* synthesis of sphingolipids occurs at the cytosolic face of the endoplasmic reticulum, and starts by the condensation of serine and palmitoyl-CoA via serine palmitoyl-transferase. The resulting keto-sphinganine is reduced to form sphinganine, and N-acylated by ceramide synthase to dihydroceramide, which is then desaturated to yield ceramide (N-acylsphingosine). Ceramide can be transformed to sphingomyelin, the major sphingophospholipid, by addition of phosphocholine, or converted to glycolipids by addition of oligosaccharides. Catabolism of sphingolipids produces ceramide. Degradation of ceramide by ceramidase releases sphingoid bases, that can either be reutilized for complex sphingolipid biosynthesis or phosphorylated Phosphorylation of sphingosine by sphingosine kinase yields sphingosine 1-phosphate, which is then dephosphorylated by a lipid phosphate phosphatase or cleaved by the sphingosine 1-phosphate lyase. Ceramide, sphingosine and S1P have all been demonstrated to be second messengers, conserved from yeast to man [28].

or else through binding specific target proteins that accommodates more or less transiently on the membrane bilayer. Ceramides cause a variety of important changes in the physical properties of the cell membranes. When they are incorporated into phospholipid membranes, they increase the order of the acyl chains, cause lateral separation of domains enriched in ceramide, permeabilize lipid bilayers, facilitate membrane fusion and fission, and induce transmembrane (flip-flop) lipid motion [40]. This explains that ceramide signaling occurs mainly in localized domains in the cell plasma membrane that are rich in sphingolipids.

Ceramide is produced mainly by two ways: *de novo* synthesis or decomposition of sphingomyelins by sphingomyelinases (Fig 2.10). The sphingomyelinases are the most heavily studied enzymes in sphingolipid metabolism. Currently, five distinct enzymes have been identified based upon their pH optima, cellular localization, and cation dependence. The neutral membrane-bound Mg^{2+}-independent sphingomyelinase (N-SMase) and the lysosomal acid pH optima sphingomyelinase (A-SMase) have been the best studied for their roles in ceramide generation [29]. An increase in N-SMase activity, a subsequent decrease in sphingomyelin, and an increase in ceramide have been demonstrated in response to TNFα, Fas ligand, chemotherapeutic agents, heat stress, ischemia/reperfusion, and interleukin-1. In addition, both arachidonic acid and glutathione depletion have been shown to activate this enzyme [9, 10-12]. In *de novo* synthesis, condensation of serine and palmitoyl CoA by serine palmitoyltransferase (SPT) initiates the pathway and generates ketosphinganine which is then reduced, N-acylated, and desaturated by addition of the 4-5 trans double bond in a series of enzymatic steps, leading to the generation of ceramide.

The use of "short-chain" ceramides (C2-ceramide) in experimental studies is reasonable because (a) their physical and chemical properties are similar to those of the long-chain analogs, apart from their capacity to be dispersed in water, (b) targets that respond to short-chain ceramides have been shown to respond equally to long-chain analogs, or to sphingomyelinase treatment, and (c) short-chain ceramides are actually natural constituents of mammalian cells.

Short-chain ceramides, which do not give rise to ceramide-rich lateral domains, are used experimentally in very high concentrations, and they are metabolized but slowly. Consequently, their high local concentration in the membrane overcomes the need for ceramide-rich domains.

2.5 Sphingolipids and Nervous System

Studies in central and peripheral nervous systems have shown that the responses to sphingolipid metabolites can be very complex, but the factors that influence the final response are slowly being identified. Recent experiments have shown that the intracellular effects of a particular sphingolipid metabolite depend on the cell type and stage of the cell development, as well as on the ratio between different metabolites and on the specific subcellular compartment of generation [41–43].

To better understand the role of sphingolipid mechanism in the nervous system, several sets of studies has been done. It was observed that, in neuronal tissues ceramide level is significantly higher than other tissues, also in normal and pathological conditions [44].

Ceramide is produced in the cells in alternative ways. The pool of ceramide generated by the activation of sphingomyelinases (SMases) has long been involved in the signaling functions of ceramide. This pathway leads rapid increase of cellular ceramide in response to diverse stimuli. On the other hand, ceramide generated from *de novo* pathway has been recognized much later as a second messenger. These two lines of ceramide production are the main sources of ceramide. Contribution of both pathways in the generation of bioactive ceramide has been investigated in many cells. In central nervous system, ceramide and sphingolipid derivatives have different type of action mechanisms. Neurotrophins are one of the important binding molecules which indirectly results in ceramide production. Neurotrophin binds to neurotrophin receptor (p75NTR), stimulate sphingomyelin hydrolysis and lead to subsequent ceramide elevation. That discovery highlighted the importance of ceramide in the regulation

of death and survival in the system [45]. Many studies have identified the effects of exogenously added ceramide analogues on cultured cells, and in cultured neurons both outgrowth and survival/death effects have been reported [46, 47]. It was demonstrated that in cerebellar granule cell culture, exogenously applied short chain C2-ceramides or SMase results in apoptosis [48, 49]. Also, nerve growth factor (NGF)-induced activation of N-SMase through p75NTR generates the pool of ceramide that mediates glutamate release [50]. On the other hand, inhibition of ceramide synthesis resulted in a decrease in cell survival accompanied by an induction of apoptotic cell death and dendritic differentiation of Purkinje cells with no detectable changes in other cerebellar neurons. Cell-permeable ceramide acted synergistically with the neurotrophin family, which has been previously shown to support Purkinje cell survival. These observations suggest that ceramide is a requisite for the survival and the dendritic differentiation of Purkinje cells [46].

Several lines of evidence have recognized ceramide and its metabolites as important second messengers in hippocampal neurons. The effects of ceramide on hippocampal neuron survival depend on the developmental neuronal stage and the concentration of ceramide used. Hippocampal neurons express also p75NTR as well as TrkB and TrkC. Consequently, NGF is able to activate p75NTR without activating Trk, and to increase ceramide levels by activation of N-SMase. This, in turn, leads to significant activation of axonal growth during the first day in culture [51]. At concentrations below 5 μM exogenous ceramide either does not affect survival during the first day in culture [52] or increases cell viability [47]. In addition, ceramide concentrations lower than 1 μM protect hippocampal neurons from several insults such as excitotoxicity, $FeSO_4$ and amyloid β-peptide [53]. At concentrations over 5 micromolar exogenous ceramide given to immature hippocampal neurons causes apoptosis [52]. In mature hippocampal neurons even low concentrations of ceramide causes cell death. Contrarily, dendritic growth in hippocampal neurons requires ongoing sphingolipid synthesis. Upon incubation with fumonisin B1 (FB1), an inhibitor of acylation of sphingoid long-chain bases, dendritic growth rates are 25% slower than those of control cells, resulting in neurons with shorter dendritic arborization and less dendritic branch points per cell, and readily apparent differences appears in the morphology compared to control cells after

10-14 days in culture [54]. Hippocampal neuronal death is responsible for memory dysfunction and is characteristic of some neurodegenerative diseases such as Alzheimer's disease. Paralelly, increased levels of ceramide has been found in the nervous system, particularly in the hippocampus of patients of AD [55, 56]. Also, shingolipids have emerged as significant regulators of amyloid-β production which is essential in AD.

3. MATERIALS AND METHODS

3.1 *In Vitro* Brain Slice Preparation

Sprague Dawley rats (p12-p24) were decapitated by guillotine. After opening the skull, brain is rapidly removed (around 40-60 seconds), and immediately transferred to ice-cold carbogenated artificial cerebrospinal fluid (ACSF) containing NaCl 125 mM, KCl 3.75 mM, $CaCl_2$ 2mM, $MgCl_2$ 1mM, NaH_2PO4 1.25 mM, $NaHCO_3$ 26 mM, D-glucose 10 mM. The ACSF was constantly equilibrated with carbogen (95% O2 - 5% CO_2) and were kept at pH 7.4. The block of brain tissue was sectioned into 200 μM transversal slices by using vibroslicer (Vibroslicer, Campden Instruments, UK). Slices were transferred into a beaker and put on a grid filled with carbogenated ACSF and incubated at 30-33 C° for 60 minutes. After incubation, a slice was transferred to a submerged-type constant flow recording chamber perfused at a rate of about 1.0-2.0 ml/min with carbogenated ACSF at room temperature. The experimental and surgical procedures were approved by the Animal Research Committee of Boğaziçi University

3.2 Whole Cell Patch-clamp Recording

Patch-clamp recordings were obtained from the soma of the pyramidal neurons in the CA1 layer of the hippocampus by using a patch-clamp amplifier, EPC-7 (List Medicals, Darmstadt, Germany). Analog signals were filtered through a 3 kHz Bessel-filter and converted into digital signals by ITC-18 A/D converter (Instrutech, Washington, USA) at 25 kHz. Strathclyde-WCP (Dr. J.Dempster) was used for data acquisition, Igor-Pro 5.0 was used for data analysis. A stimulus isolation unit, Iso-Flex (A.M.P.I., Jerusalem, Israel) was used to generate constant current pulses for stimulation. A borosilicate recording electrode (pipette resistance changes between 4-7 MΩ) was prepared by using a microelectrode puller PP-81 (Narishige, Tokyo, Japan) from borosilicate capillaries (Hilfenberg, Malsfeld, Germany). Pipettes were filled with in-

tracellular solution. Intracellular solution contained the following: CsF 100 mM, CsCl 40mM, EGTA 10 mM, HEPES 10 mM, $CaCl_2$ 1mM (pH 7.3). Cs^+ was used as the main cation to substitute K^+ ions and to suppress potassium conductances (including that generating the slow inhibitory postsynaptic potential; sIPSP). Also, fluoride is used as main anion which blocks the chloride conductance. Intracellular fluid was filtered through 0.2 μM membrane filter in order to remove any clogging particle in the solution and kept at -20 °C in 2 ml aliquots. For each experiment intracellular solution was thawed. Brain slices were viewed by two upright objectives. The objective with 4x magnification is used to view the whole slice, to localize the region of interest and to insert the stimulation electrode on to the Schaffer collateral pathway. Water immersion objective with 60x magnification was used to identify the cells specifically to obtain recordings.

Under 4x objective, stimulation electrode was placed on Schaffer collateral fibers at a distance of 100 μM from the cell body layer of CA1 pyramidal cells in order to stimulate mainly proximal apical dendritic synapses. Electrical stimuli was applied as constant current pulses of 0.1 ms at 0.1 Hz to the slice extracellularly through a tungsten bipolar stimulation electrode. After inserting stimulation electrode, objective was switched to 60x water immersion objective, and under 60x objective, pyramidal cell soma at CA1 region of the hippocampus was visually localized. In a stepwise manner, patch pipette was brought closer to the cell soma. When pipette was in the bath, positive pressure was applied on to the pipette through a syringe connected to a tubing in order to prevent pipette tip clogging. 10 mV voltage pulses was applied on to the pipette from a function generator (GFG-9019) in order to follow the changes in pipette resistance and capacitance and to observe seal formation between the pipette and the cell. Ground electrodes were chlorinated before recording in order to decrease liquid junction potential. Offset removal was performed after entering the bath by using the offset removal knob on the amplifier.

The pipette touched the cell soma very gently, in parallel; around 20-30% decrease in current amplitude was observed in the scope. Then the pressure was immediately released and a slight negative pressure was applied. Current amplitude fell

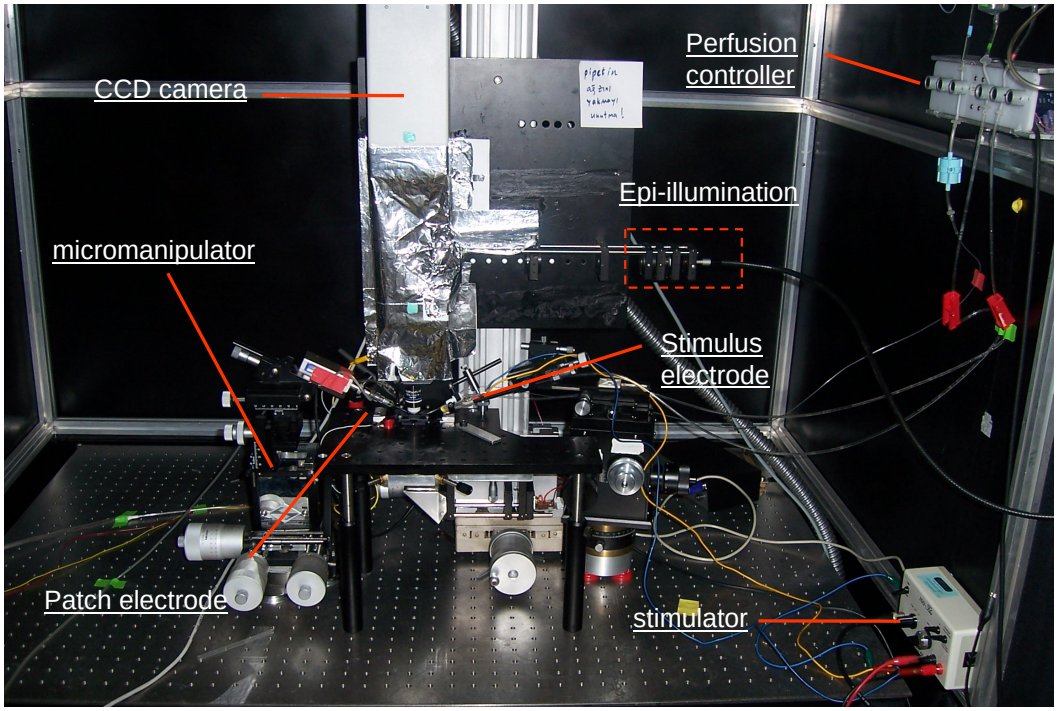

Figure 3.1 Setup for electrophysiological recording and fluorescence imaging.

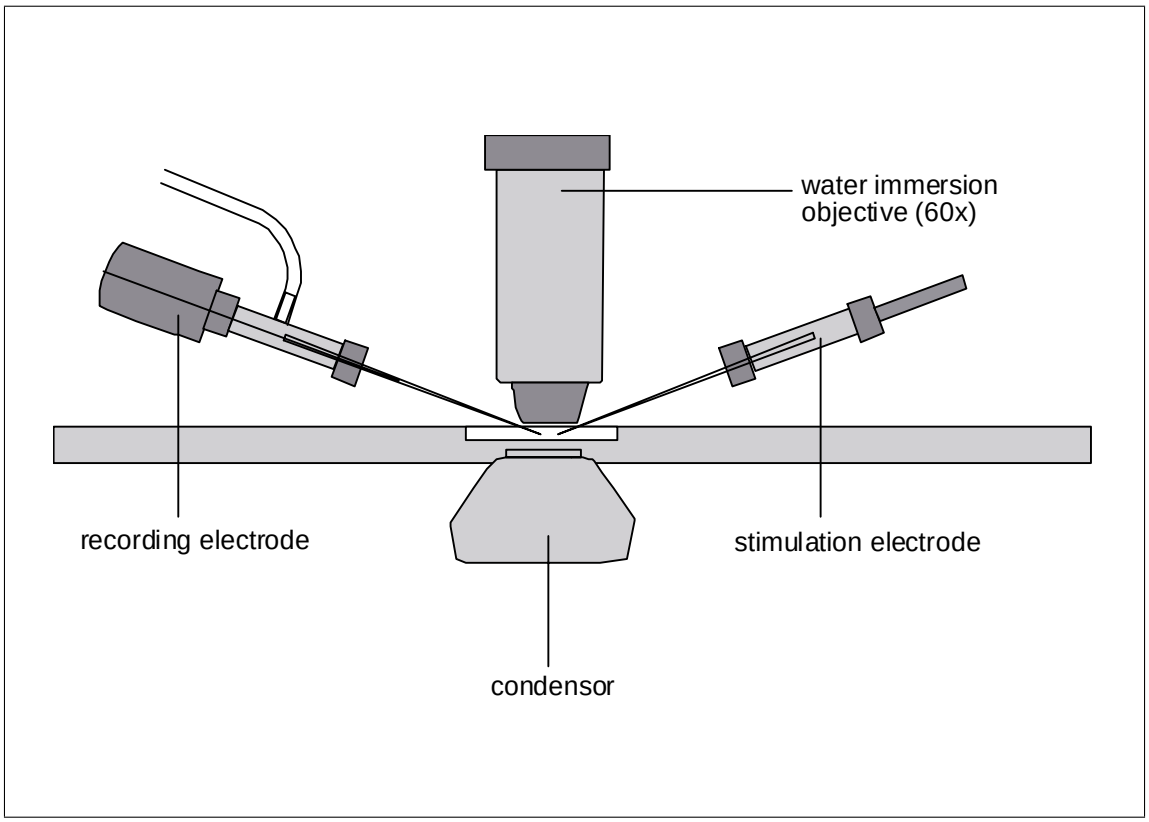

Figure 3.2 Positions of the stimulation and recording electrodes.

into baseline level, only leaving capacitive currents resulting from the pipette interface. That step, caused an increase in resistance between the pipette tip and membrane, around gigaohm; and tight seal (gigaseal) was formed between pipette tip and membrane. A brief suction was applied and resulted with break-in of the membrane, and formed whole cell configuration. Fast and slow capacitive currents were compensated electronically. During seal formation and break-in, command potential was clamped at -60 mV. After break in, during continuous recording, membrane potential was clamped at -50 mV. In order to obtain postsynaptic currents, a current stimulus (0.1 ms) from stimulus generator was applied and excitatory post-synaptic currents were recorded. Each current was acquired in 500 ms sweeps.

All the chemicals in ACSF were prepared immediately before each experiment from the frozen stocks and were administrated via bath solution. Kynuronic acid were used to block whole glutamatergic response of the cell. d-2-amino- 5-phosphonopentanoate (AP5) and 7-Dinitroquinoxaline-2,3-dione (DNQX) were used in the experiments to antagonize NMDA receptor- and AMPA/kainate receptor-mediated currents. In the composition of the intracellular solution, Cs^+ was used in order to substitute K^+, and F^- was used to block Cl^- channels. N-Acetyl-d-sphingosine (C2-ceramide, a membrane-permeable form of ceramide) was dissolved in dimethylsulfoxide (DMSO) and kept as 10 mM stock at -20 °C. The final concentration of DMSO during perfusion did not exceed 0.1%.

3.3 Fluorescence Imaging of the Pyramidal Neurons

Fluorescence imaging were performed to visualize the morphology of the CA1 pyramidal cells. An epi-fluorescence system was used. For excitation, Xenon-Arc lamp (250 nm-1000 nm) was used as a light source. Excitation-emission filter block was used to filter certain wavelengths during excitation and emission (excitation: 425 nm, emission: 528 nm) Lucifer yellow dye was used for morphology staining. Patch pipette was filled with fluorescent dye, Lucifer yellow (10 μM). Cells were stained via patch pipette, after rupturing of the neuron membrane under the electrode. Excitation-emission spec-

trum of the Lucifer yellow stain was consistent with filter block (excitation: 425-430 nm, emission: 515-540 nm). 15-30 minutes after breaking-in the membrane, excitation light source was turned on, contrarily transmission light through the condenser were turned off and under high exposure, pyramidal neurons emitting light was captured through 12 bit high speed CCD camera, SensiCam QE (PCO Imaging, Kelheim, Germany).

4. RESULTS

4.1 Whole-cell Current Recording from CA1 Pyramidal Cells

4.1.1 Effect of C2-ceramide on NMDA and non-NMDA currents

Schaffer collateral stimulation evoked CA1 pyramidal single cell current response was recorded by patch-clamp tight seal whole cell recording technique. Whole cell currents were recorded from the soma of the hippocampal pyramidal neurons. A single brief current pulse (0.1 ms) delivered to the Schaffer collateral pathway evoked large inward postsynaptic currents. Stimulus control was regulated in order to obtain the optimal constant current from the stimulator during the recording.

With a delay of 50 ms a stimulus was delivered to Schaffer collateral and evoked a current response at - 50 mV (fig. 4.1).

At -50 mV, a depolarizing current on the membrane results in a relief of Mg^{2+} block on NMDA channels; so NMDA component could be observed in the plot. Current has an early component which signifies an initial peak of current of great amplitude and a second late component signifies a slow inactivation. After applying kynuronic acid (1 mM), inward current was totally blocked (each plot is an average of 10 sweeps). NMDA currents were isolated to record the effect of ceramide on NMDA receptor activity. In order to verify the existing components of the glutamatergic current as NMDA and non-NMDA; pharmacological applications were done.

Addition of DNQX (12 μM) significantly lowered the amplitude of the current. Fast activation phase was disappeared and slow activation and inactivation phases were left. The remaining current is an NMDA current which shows a slow activation phase followed by a slow falling phase. NMDA current was blocked by AP5 (60 μM) (fig 4.2).

C2-ceramides (10 μM) were tested on NMDA current response. At the end of 300 s of C2-ceramide application, NMDA current amplitude was lowered to 77 $\pm$8 % (mean $\pm$ stdev) (n=5) of its original level (paired Student's t test; $p < 0.05$). NMDA current was blocked with AP5 application (plot was not shown) (fig 3.3). NMDA current was depressed around 23 % after C2-ceramide application.

In order to check the effect of DMSO on the current, DMSO was applied into the bath. NMDA currents were recorded. At the end of 300 seconds, there was not a significant change in NMDA amplitude ($n = 5$) (paired Student's t test; $p > 0.05$) (fig 3.4).

Non-NMDA currents were isolated by applying AP5 (60 μM) at -50mV. The resulting current amplitude composed of a very fast activation phase and a relatively fast falling phase. When AP5 was applied, amplitude was not changed significantly, however a slow component on the falling phase was disappeared (fig. 3.5). When C2-ceramide was added to the bath, there was not any significant change in the non-NMDA current amplitude. After 300 seconds of C2-ceramide application, it was observed that non-NMDA amplitude was 92% $\pm$7 (mean $\pm$ stdev) of its original level ($n = 3$) (paired Student's t test; $p > 0.1$) (fig. 3.6).

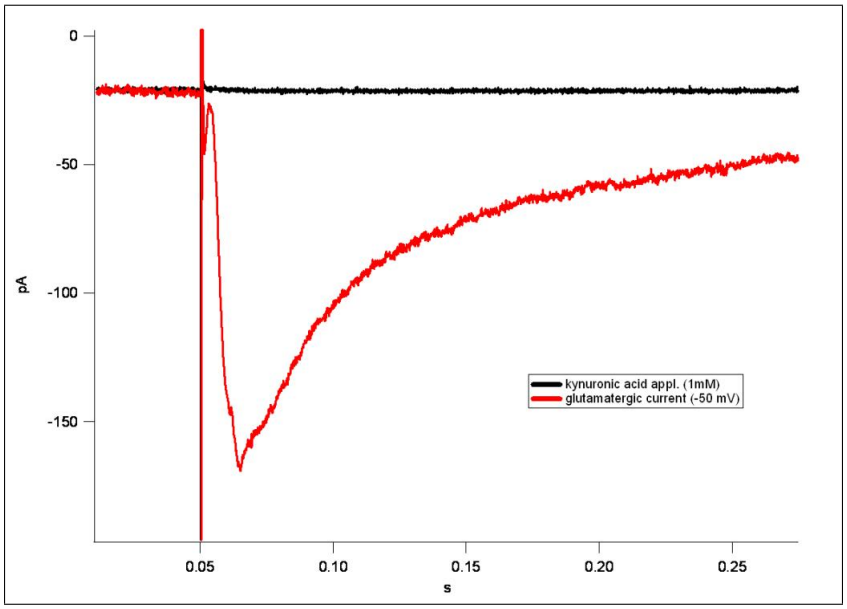

Figure 4.1 Glutamatergic currents and their inhibition. Total glutamatergic response of the cell was blocked after kynuronic acid application (1 μM).

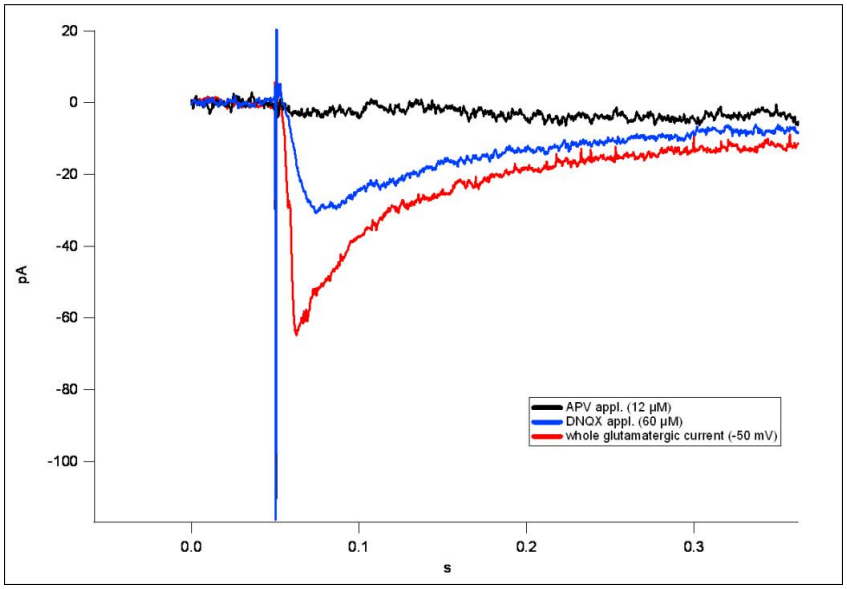

Figure 4.2 Isolation of NMDA current. NMDA current was isolated from total glutamatergic current by application of DNQX (12 μM). NMDA current was blocked by AP5 (60 μM) with its selective antagonist.

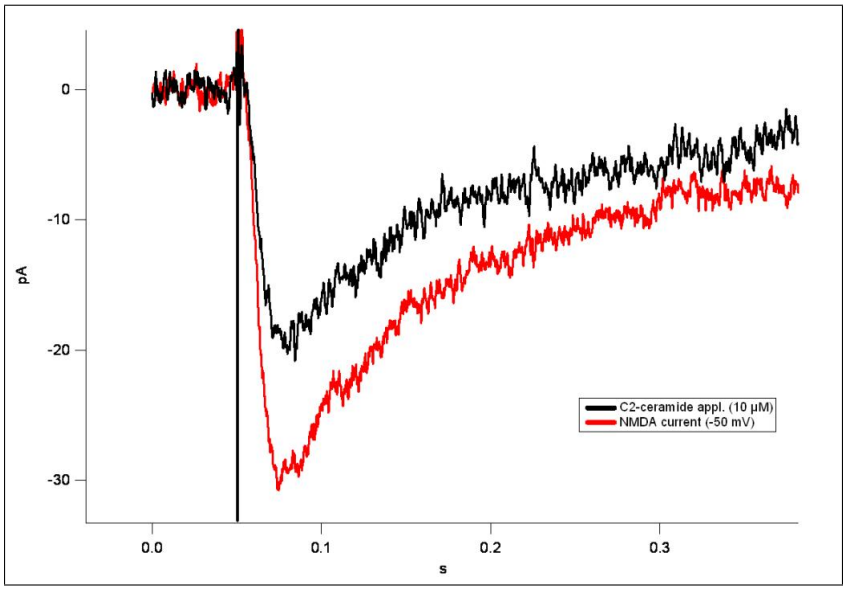

Figure 4.3 Modulatory effect of C2-ceramide (10μM) on NMDA current.

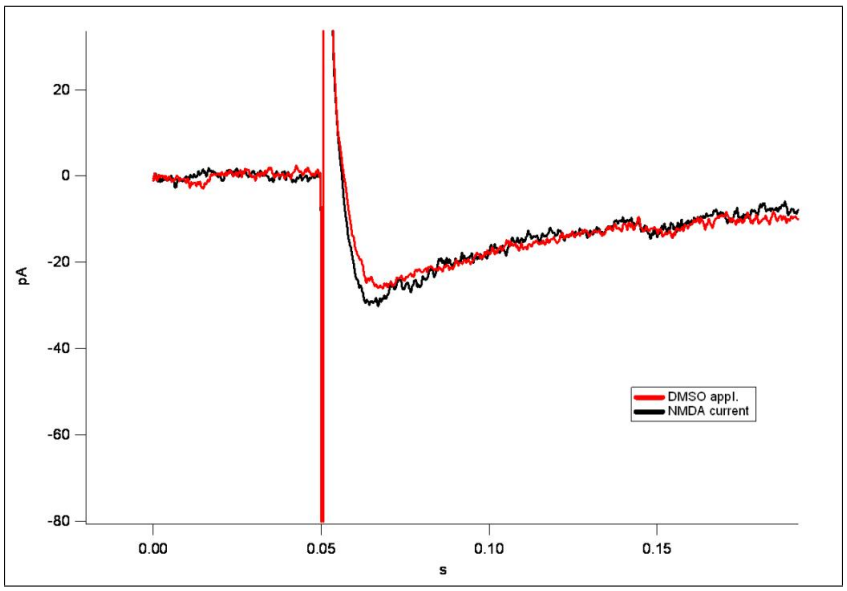

Figure 4.4 Control of the effect of DMSO on NMDA current

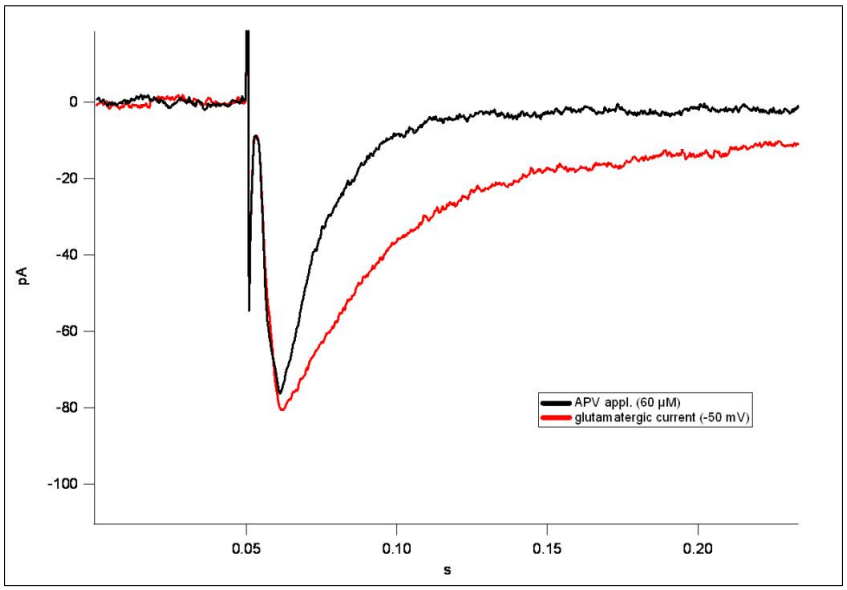

Figure 4.5 Isolation of non-NMDA currents. non-NMDA current was isolated from total glutamatergic current by application of AP5 (60 μM).

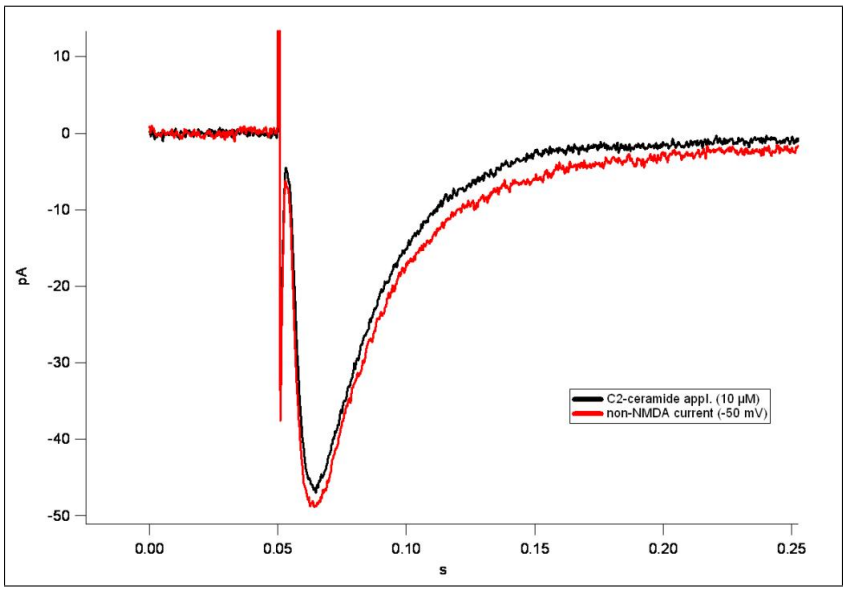

Figure 4.6 Effect of C2-ceramide (10 μM) on non-NMDA current.

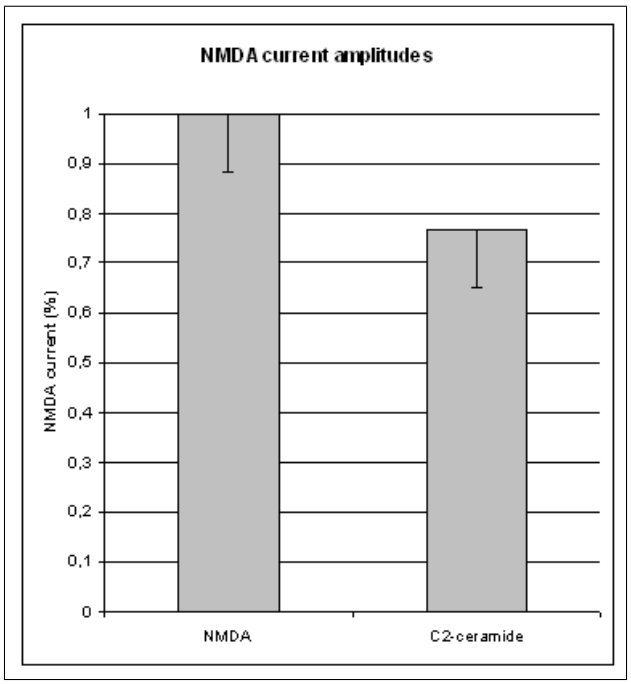

Figure 4.7 Change in NMDA current after C2-ceramide application.

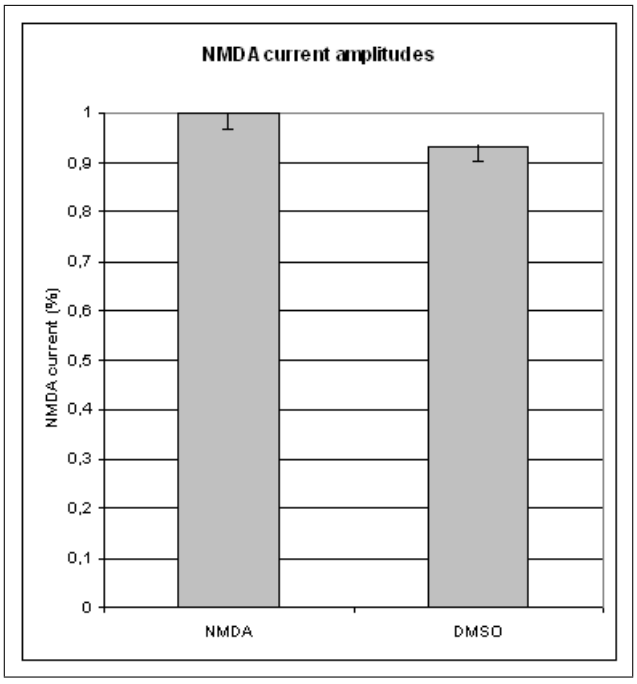

Figure 4.8 Change in NMDA current after DMSO application.

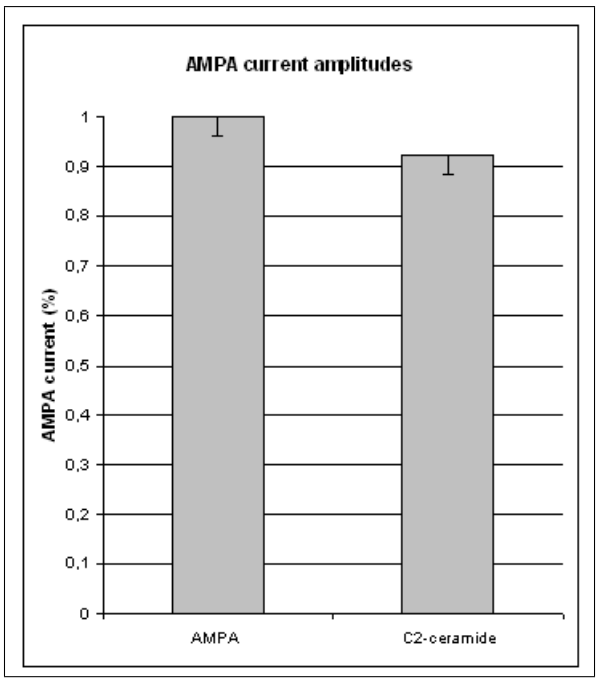

Figure 4.9 Change in non-NMDA current after C2-ceramide application

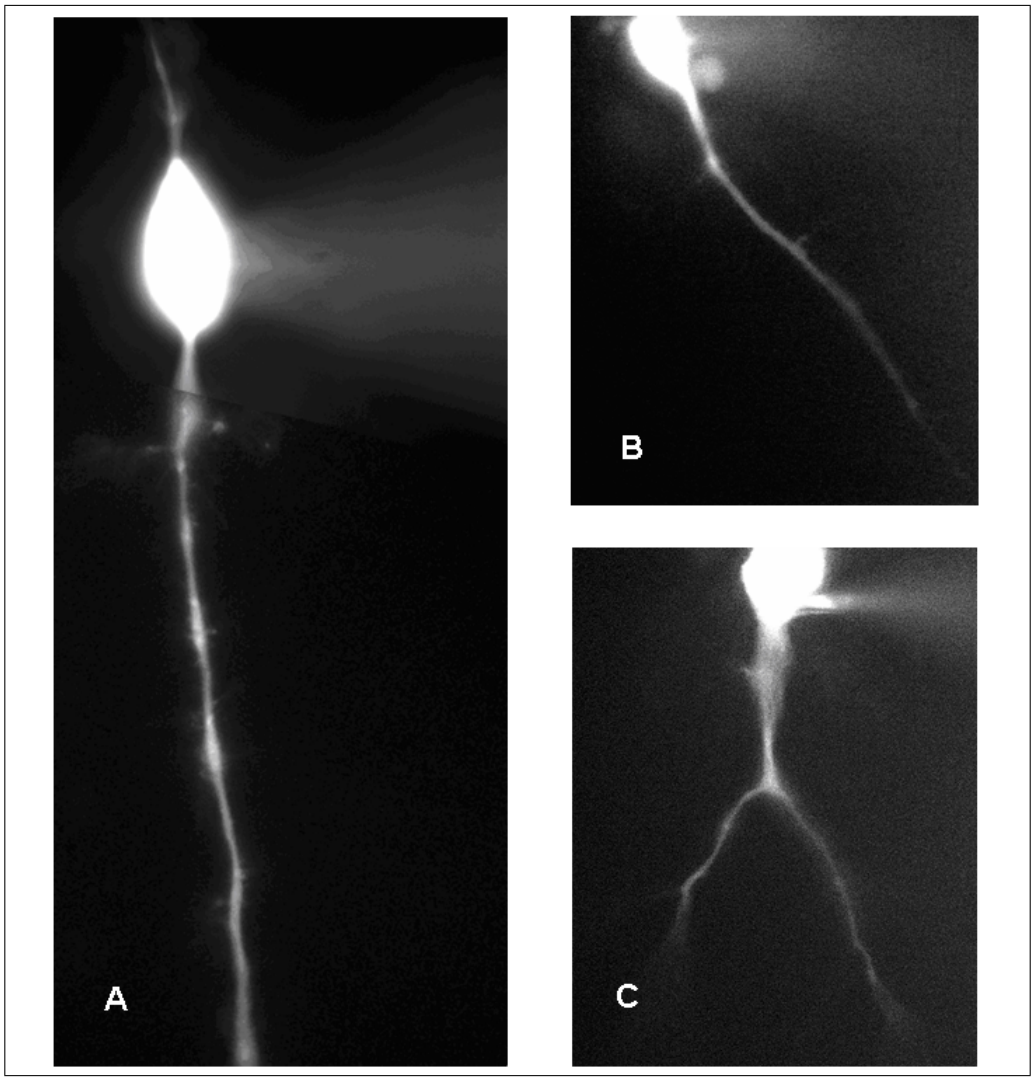

Figure 4.10 Fluorescence images of pyramidal neurons. A, B: Lucifer yellow diffuses all through the pyramidal cell, soma and dendrites (downwards) are visible. C: Dendritic split is visible at the bottom side.

5. DISCUSSION

5.1 Excitatory Post-synaptic Currents and Their Role in Synaptic Transmission

Majority of our knowledge about physiology and pharmacology of synaptic transmission in the central nervous system comes from studies on the hippocampus. In this pathway, excitatory amino acids are essential for synaptic transmission. The Schaffer collateral, among the others, is the best-studied synaptic pathway in the hippocampus. This pathway constitutes a monosynaptic projection from CA3 region to CA1 pyramidal neurons in the hippocampus. This projection makes connection to CA1 region where there is highest density of glutamate receptors. Electrical stimulation of the Schaffer collaterals in stratum radiatum results in the sequence of excitation-inhibition. Glutamate has a critical place in synaptic transmission in hippocampus. Glutamate released into the synaptic cleft binds to post-synaptic NMDA as well as non-NMDA receptors and evokes a synaptic response which is a post-synaptic excitatory current. The function of post-synaptic iGluRs is to mediate fast excitatory synaptic transmission. Glutamate receptors comprise the glutamate receptor sites on their surface, the elements that make the ionic channel selectively permeable to cations, as well as all the elements necessary for interactions between different functional domains. Thus, the glutamate receptor sites and the cationic channel are part of the same unique protein. NMDA, AMPA and kainate receptors are co-expressed in many neurons. When the membrane potential is close to the resting potential of the cell (i.e. -60 mV), a large fraction of the NMDA receptors are blocked by Mg^{2+} ions. Therefore glutamate first activates non-NMDA receptors.

The non-NMDA mediated depolarization is necessary to activate the NMDA receptors. AMPA receptors is permeable to sodium and potassium, as main cations. This AMPA-receptor mediated EPSC generates a fast-rising and fast-decaying EPSC. When kainate receptors are present in the post-synaptic element, they are activated

once glutamate is released in the cleft. They allow a transient Na^+ influx (inward current) and sometimes a Ca^{2+} influx, through the post-synaptic membrane (inward current). This kainate receptor-mediated EPSC is smaller and slower (time to peak of the order of 5-10 ms) than the AMPA-mediated one, thus giving a slow-rising, low amplitude EPSP component. NMDA type receptors open more slowly and remain open longer than AMPA receptors. They are also regulated by voltage-dependent block by Mg^{2+}. As a result they can be activated only when the membrane is sufficiently depolarized to remove this block. NMDA receptors act as a coincidence detector. In this condition, membrane current fluxes only when agonist binding and cell depolarization take place simultaneously. When they open, they allow a large Na^+ and Ca^{2+} influx through the post-synaptic membrane. The resulting EPSC triggers a slow-rising, long duration current and the resulting increase of intracellular Ca^{2+} concentration triggers a cascade of molecular events in the post-synaptic cell.

In our research, the pyramidal cells of hippocampal CA1 region is the region of our interest. The monosynaptic projection from CA3 to CA1 is apparent and the evoked currents, excitatory post-synaptic currents were elicited by electrical stimulation of the Schaffer collateral. Each neuron, located in the CA1 region, is in direct contact with axons of the CA3 pyramidal neurons. Stimulation frequency and intensity is the critical parameters in the formation of post-synaptic current. We observed relatively low-amplitude glutamatergic currents when the stimulation electrode was located long distance from patched neuron. So, we inserted at a distance within 100 μm from the cell body layer of CA1 pyramidal cells in order to stimulate mainly proximal apical dendritic synapses, thereby minimizing the space-clamp errors and dendritic filtering. In order to elicit a stable EPSC recording, the stimulus intensity was adjusted to obtain 50 percent of maximum current amplitude. Depolarizing the neuron by keeping the membrane holding potential at -50 mV and stimulating the Schaffer collateral pathway was sufficient to evoke the glutamatergic response. In order to isolate EPSC components, selective antagonists of the NMDA and non-NMDA were used. When kynuronic acid was applied to the perfusion system, glutamatergic response was totally disappeared. It is known that kynuronic acid blocks the co-agonist binding site of the iGluRs [57]. As the glycine is the co-agonist of the glutamate receptors,

any block on glycine binding site will totally abolish the total glutamatergic response. Our prediction was to elicit particularly excitatory synaptic currents along the CA1 region without any epileptiform activity. Our synaptic output was consistent and the stimulation of the Schaffer collateral at 0.1 Hz, (close to the physiological conditions) resulted in the generation of single EPSC. Absence of a system of recurrent excitation in the CA1 region is an important consideration. The presence of an extensive system of recurrent excitation in the CA3 region is one of the reasons why disinhibition tends to cause epileptiform activity in this area. In disinhibited slices, polysynaptic excitatory responses are commonly elicited in the CA3 region by afferent stimulation, but this is less common in the CA1 region. This is the reason why we obtained the recordings from CA1 as being the output region of hippocampus to entorhinal cortex [58, 59]. Glutamatergic currents are composed of NMDA and non-NMDA currents. In our experiments, NMDA and non-NMDA currents were studied. NMDA currents were isolated by blocking non-NMDA component with competitive antagonist, DNQX. Blocking of non-NMDA current results with a dramatic decrease in current amplitude and the remaining current showed a slow rising and recovery phase. It was verified that the recorded current was NMDA current by using AP5, which is a selective blocker of NMDA receptor. After application of AP5 (60 μM), the NMDA current was totally blocked. AP5 is a competitive antagonist, binds on the ligand site and totally blocks the receptor activity.

5.2 Sphingolipid Metabolism and Modulation of Membrane Currents

Sphingolipids are in association with signaling pathways and effective in the fate of the cell. The action mechanism of the sphingolipids depends on the cell type, age and the stress factors effective on the cell metabolism [60].

Ceramide is a key molecule in the sphingolipid metabolism. It is involved in the signaling cascades of the cell, and has been shown to mediate growth arrest,

differentiation and apoptosis. Previously, membrane permeable ceramide molecules have been utilized in different studies on different cell types and its role in modulating the membrane currents has been investigated [32]. Cytokines, ionizing radiation, heat shock, chemotherapeutic agents, exposure to receptor-specific ligands (TNFα, Fas ligand, 1,25-dihydroxyvitamin D3), and environmental factors such as stress, hypoxia/reperfusion lead to ceramide production via activating sphingomyelinases. Consequently, ceramide production mediates several cellular activities via kinases and phosphatases in the cell [61]. Those enzymes act on several proteins in the cell, as well as functional membrane proteins, such as ion channels. There are several reports on the role of ceramide in modulating the membrane ion fluxes. It was shown that, ceramide acts as an intracellular messenger that activates the transcription factor NF-kappaB, and mimicked the actions of tumor necrosis factor (TNF) on Ca^{2+} current density and currents induced by glutamate receptor agonists [62]. Yang also has showed that ceramide activated protein phosphatases lead to the depression in the synaptic currents mediated by the ionotropic glutamate receptor [63]. Furthermore, ceramides are effective in blocking the inward rectifier IK and/ or Ca^{2+}-activated K^{+} [BKCa] in a number of cells including oligodendrocytes, neuroblastoma and GH3 cells and sensory cells. Ceramides have a key role in mediating these cascades of events in the cell. It is well documented and also different lines of studies continue on ceramide role in mediating intracellular pathways as a secondary messenger.

In our study, we investigated the role of ceramide on the excitability of the neurons in the hippocampus. Hippocampus is composed of principal excitatory neurons which carries ionotropic glutamate receptors in fast synaptic transmission. NMDA and non-NMDA receptors are colocalized in the membrane and mediate ionotropic currents during synaptic transmission. According to our results, we observed that C2-ceramide (10 μM) have depressed the NMDA currents by 23 %. However non-NMDA component of the glutamatergic currents did not show any significant change with ceramide application. Vehicle (DMSO) control recordings also did not show any significant change in NMDA amplitudes. These results showed that application of C2-ceramide resulted with a depression in NMDA currents; however, in non-NMDA currents, there was not any significant change. We have also checked whether DMSO has any effect on

glutamatergic currents or not. DMSO was used to solubilize C2-ceramide. DMSO concentration did not exceed 0.1 % in the final ACSF in our experiments. According to our results, there was not any significant change in current levels so we can assume that there is not any effect of DMSO on NMDA current.

In order to check whether ceramide effect was reversible or not, the slices were washed with ACSF, however the original NMDA response was not recovered. We also investigated the non-NMDA receptors, AMPA and kainate by blocking NMDA receptor. In these experiments the non-NMDA current amplitude did not show any significant change. Our first implication was that, C2-ceramide specifically alters the ion flux through NMDA receptors. Previously, Yang has shown that glutamatergic currents were sensitive to ceramide, which activates protein phosphatases [63]. However in our experiment, as a result of ATP, GTP depletion in the cell, and the absence of ATP in the intracellular solution, the intracellular mechanisms were not driven. This implication has put forward the question of "how NMDA receptor ionophore complex was blocked". It was proved that there are several routes to block or modulate the activity of NMDA channel. In resting state membrane potential, in channel pore region there is a voltage dependent Mg^{2+} block on the channel. N site of segment M2 constitutes the Mg^{2+} block site, and that segment M2 contributes to the lining of the ion channel pore of the NMDA receptor channel. NMDA receptor channels are also blocked by physiological concentrations of intracellular Mg^{2+} in a voltage-dependent manner [17]. The block by intracellular and extracellular Mg^{2+} is suggested to be mediated by different sites in the channel. Drugs such as MK-801, dissociative anesthetics, phencyclidine and ketamine binding sites are within the electric field of the channel and for the blockage of the channel with those drugs, the channel should previously be opened by agonist. At allosteric modulation sites, pH, polyamines are efficient. At redox sites, redox agents, nitric oxide have modulatory roles. Also zinc, ethanol and arachidonic acid have a role in modulation NMDA currents [64–66]. Apart from those, there are intracellular modulation mechanisms on NMDA channel-receptor complex. Protein phosphorylation and dephosphorylation are thought to be important mechanisms for the regulation of synaptic activity. Extracellular ceramide application may mediate several events intracellularly [28].

Besides the intracellular cascades, its direct effect on membrane is a point of discussion. Plasma membrane of many cell types, including neurons and glia, contains lipid rafts, that are specific microdomains implicated in several cellular processes including signal transduction, membrane trafficking, cytoskeletal organization, and pathogen entry. It has been reported that the relative proportion of ceramide and phospholipids plays a large role in the formation of ceramide-enriched microdomains and the way in which ceramides perturb the structure of bilayers. The generation of ceramide within small rafts alters the biophysical properties of rafts and triggers the fusion of small sphingolipid-enriched rafts to larger ceramide-enriched signaling platforms. Ceramide-enriched membrane platforms serve to trap and cluster receptor molecules, e.g. CD95, an event that is required for apoptosis induction by CD95 [67]. In addition to receptor molecules, membrane platforms seem to contain many signaling proteins and even some voltage-gated K^+ channels have been shown to associate with lipid rafts. Transmembrane proteins can also make partition into lipid rafts, but the mechanism of their raft association is unclear. Differential affinity for rafts leads to compartmentalization of specific proteins in the plane of the membrane. In addition, rafts are important for neuronal adhesion, axon guidance and synaptic transmission. In this model, ceramide-enriched membrane platforms has been associated with several membrane proteins including ion channels. Voltage-gated K^{2+} channels have been shown to associate with lipid rafts [68]. It was also reported that treatment of the jurkat cells with C16-ceramide was resulted with clustering of Kv1.3 within ceramide-enriched membrane platforms and inhibition of the channel's activity. Mechanism of this inhibition can be explained as the transformation of small rafts into large ceramide-enriched membrane platforms upon stimulation via CD95 or other receptors that trigger an activation of the ASM and a release of ceramide within small rafts, blocks Kv1.3 activity. The transformation of small rafts into ceramide-enriched membrane platforms is mimicked by addition of exogenous sphingomyelinase or C16-ceramide that also inhibit Kv1.3. In this respect, addition of ceramide, may also be functional in mediating similar responses. Recently, it was shown that glutamate receptors (AMPA and NMDA) and post-synaptic scaffold proteins are associated with rafts in neurons [69]. This relationship was also pointed out in a study investigating the increased phosphorylation and redistribution of NMDA receptors between synaptic lipid rafts and post-synaptic

densities following transient global ischemia in the rat brain [70]. Presence of lipid rafts in the post-synaptic sites of neurons has been mentioned. As a result of microdomain formation, it may well play important roles for the maintenance of post-synaptic structures, like ion channels [71]. It was also reported that, cholesterol perturbing agents inhibit NMDA-dependent calcium influx in rat hippocampal primary culture. These results suggest that the NMDA receptor can be located in cholesterol-rich membrane microdomains or alternatively that the mechanisms coupling their dynamics in the post-synaptic membrane are dependent on the integrity of the microdomains [72].

Our first results imply that ceramide has a specific modulatory effect on NMDA currents. On the contrary, AMPA receptors did not show any change in their activities. NMDA receptor's sensitivity to ceramide application is an important finding, that drew our attention on the distinctive properties of the receptors affinity to the ceramide. To postulate the existence of a modulatory site on the channel receptor for ceramide is a matter of discussion. In previous studies, modulation of NMDA receptor is extensively documented and it has several modulatory sites on it. In our case, the action mechanism of the ceramide block on the receptor or on the ionophore site is not known. It may be related with a direct block on the ionophore on a specific site or it can exert its modulatory effects by binding onto the receptor subunits. Till now, there is not any observation on the interaction of ceramide molecules with channel receptors extracellularly. However there are much evidences on the enzyme dependent modulation of ion channels related with signal cascades inside the cell. Ceramide modulated signals may depress the ionotropic glutamate receptors via protein phosphatases [63]. However direct blockage of the receptor ionophore complex excluding intracellular pathways has not been reported previously. Under this condition, we may also discuss the biophysical characteristics of the cell membrane and try to relate it with the conditions with ceramide exposure. It was reported that on the cell membrane there are several microdomains composed of sphingolipids and cholesterols [26]. Any disturbance on the sphingolipid carrying membrane microdomains, so called lipid rafts, may result with consequent modulation among the protein molecules residing in the lipid rafts. As previously mentioned, the composition of the stable lipid rafts may be disturbed by activating sphingomyelinases which results with ceramide production and

consequent accumulation of ceramide molecules in lipid raft. This structure is called as ceramide enriched microdomain. As we apply ceramide extracellularly, it behaves like ceramides which are decomposed by sphingomyelinases from sphingomyelins and form ceramide enriched membrane microdomains. Along with increase in ceramide in lipid rafts, the response status of the protein molecules residing in the rafts may change. As previously reported, NMDA and AMPA receptors are located in lipid rafts. Any disturbance in the lipid raft may affect receptor activity. Similarly, it was shown that NMDA receptor activity was depressed along with cholesterol perturbing agents [72]. Same conditions can be replicated with ceramide exposure, that means, ceramide may accumulate inside the raft and change the biophysical characteristics of the lipid raft and may mediate inhibition of the channel receptors, like NMDA receptor. In a recent study, it was shown that ceramide enriched domains alter the physical properties of the liquid ordered domains, decreasing their stability and viscosity and perturbing the lipid packing [73]. This assumption also can be objected by different points. First of all in early studies it was shown that non-NMDA and NMDA receptors are colocalized on the cell membrane [74]. Therefore, any biophysical change on the membrane moeities has to involve parallel effects both on non-NMDA and NMDA receptors. However in our case, non-NMDA currents are not affected by ceramide exposure. Although this controversy exists, there are also some observations where NMDA and non-NMDA receptors are located in different microdomains in the cell membrane, could be an explanation for demonstrating different responses [69].

As a result, the subunit characteristics of the receptors, their specific location in the membrane, also in the lipid raft and their interaction with the cell membrane has to be investigated in order to understand the exact response of the receptor protein when the sphingolipid composition is changed in the membrane.

6. CONCLUSION

In this study, we have investigated the roles of sphingolipids on *in vitro* rat hippocampal slices with patch clamp recording technique. We have recorded low frequency stimulated evoked currents from hippocampal pyramidal cells. We investigated the effects of short chain ceramides on the NMDA and non-NMDA current response. According to our results, there was a decrease in NMDA current amplitude subsequent to ceramide application, however there was no change in non-NMDA current amplitude.

NMDA current depression by sphingolipids is a significant observation. In previous studies, intracellular cascades triggered by sphingolipids were identified and modulation of the ion channel receptors by sphingolipids were reported. Our results indicate that beside the role of sphingolipids on mediating intracellular activities, they have a specific modulatory effect on channel receptors, particularly NMDA receptor on the membrane.

NMDA current has a critical role in the formation of synaptic plasticity, in a general scale, learning and memory formation in the hippocampus. Also it is known that sphingolipids are membrane components and their modulatory roles on ion receptor activity is an important finding in order to distinguish aberrations in neuron activity. In case of any disturbance in membrane sphingolipid components, as a result of external insults on the cell (hypoxia, irradiation etc.), may evaluate as a sign of pathological condition. This condition could be a landmark for the pharmacologist in order to make a suitable drug design which may alter the interaction of sphingolipids with channel receptors on the membrane.

APPENDIX A. Patch Clamp Technique

During the last 50 years, many new techniques were developed to extend the research on electrophysiology. Early in the research, extracellular electrodes were used to measure membrane potential. Before 1970s, intracellular electrical recording from single cells was done via capillary electrodes or via micropipette electrodes used to penetrate cells. During 1970s and 1980s, patch clamp technique was developed and improved. It enabled researchers to record from single cells and from isolated patches of cellular membrane that contained individual ion channels. Erwin Neher and Bert Sakmann were the inventors of patch clamp technique during 1980s. They were awarded with Nobel Prize in Physiology or Medicine in 1991. In this new technique, a micropipette, called a patch pipette, with a tip diameter of 1-2 μm is inserted on the cell membrane, forming an electrical seal in the order of gigaohms. It is important to keep the pipette tip surface clean in order to establish a tight contact with the cell and a subsequent negative suction on the pipette is necessary to establish the tight contact between the pipette tip and the membrane. By this way there is no current outflow to the extracellular media through the micropipette. Based on this technique, there are different type of patch-clamp configurations which are designed for different type of application: cell-attached, whole cell, outside-out and inside out (Fig A.1).

a. Cell attached recording: This configuration enables the recording of current flowing through the channel or channels present in the patch of membrane that is under the pipette and is electrically isolated from the rest of the cell. This is mainly used when the channel type in question requires unknown cytosolic factors for gating and these would be lost when patch was excised. This configuration is noninvasive, leaving the ion channel in its physiological environment, it may be used to test for possible alterations of channel properties after patch excision. Besides its advantages; cell-attached mode present two limitations: the composition of the intracellular environment is not controlled; the value of the membrane potential is not known and can only be estimated.

b. Inside-out recording: This inside out configuration is obtained from the cell attached configuration by gently pulling the pipette away from the cell, lifting the tip of the pipette from the bath in the air and putting it back into the solution. In this case, the intracellular environment is that of the bath and the extracellular one is that of the pipette. This configuration enables one easily to change the cytosolic side of the patch. It is therefore the method of choice to study the gating of second-messenger activated channels at the single channel level and also it is used when rapid changes in the composition of the intracellular environment are necessary to test. The main limitation is the loss of key cytosolic factors controlling the behaviour of some ion channels.

c. Outside-out recording: The outside-out configuration is obtained from the whole-cell configuration by gently pulling the pipette away from the cell. This causes the membrane patch to be torn away from the rest of the cell at the same time that its free ends reseal together. In this case, the intracellular environment is that of the pipette, and the extracellular environment is that of the bath. This configuration allows one easily to change the extracellular side of the patch. It is therefore often used to study receptor-operated ion channels. High quality and stable outside-out recording are difficult to obtain, because of its demands in steps.

d. Whole-cell recording: This configuration is obtained after cell-attached configuration. If a little suction is applied to the interior of the pipette, it may cause the rupture of the membrane patch under the pipette. Consequently, the patch pipette now records the activity of the whole cell membrane. This configuration enables the recording of the current flowing through the channels open over the entire surface of the cell membrane. The main limitation of this technique is the gradual loss of intracellular components (such as second messengers), which will cause the eventual disappearance of the responses dependent on those components.

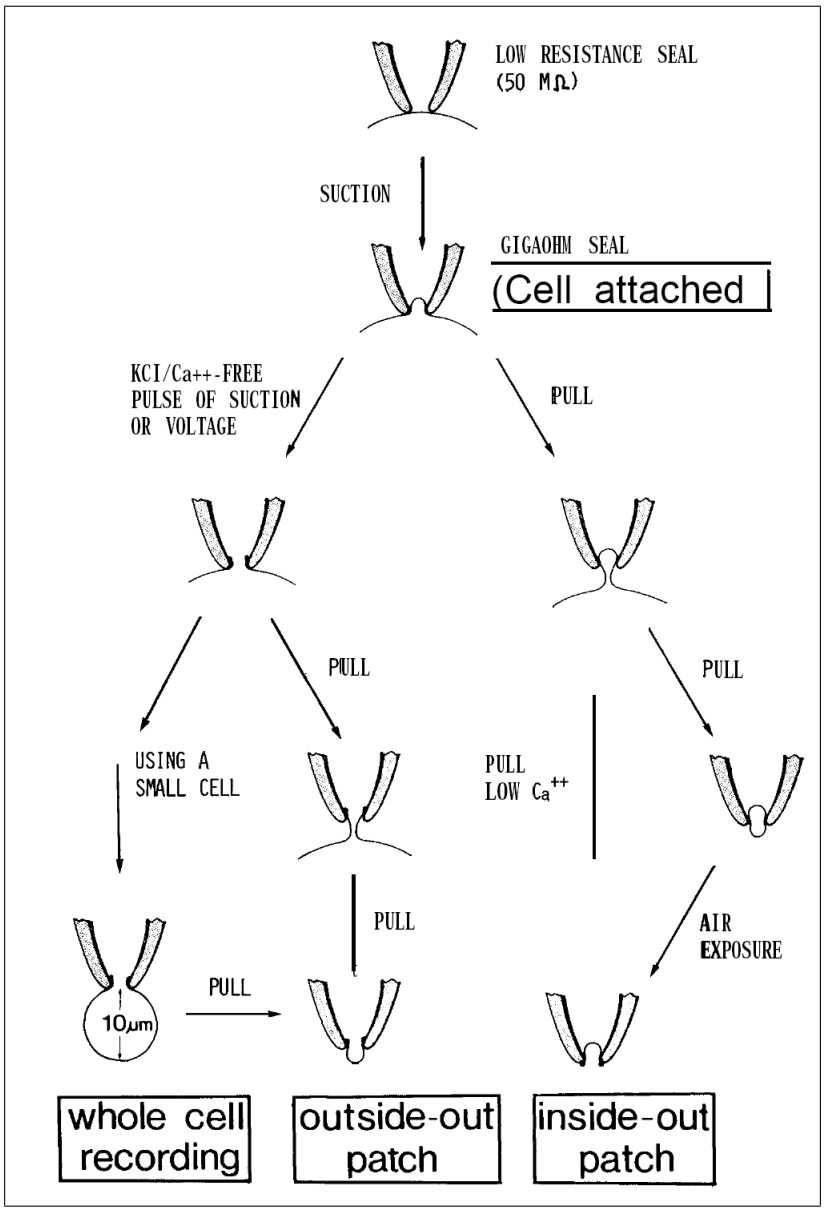

Figure A.1 Schematic representation of the procedures that lead to the different patch clamp configurations (from Nobel Lecture by Erwin Neher, 1991).

APPENDIX B. Fluorescence Microscopy

Using the phenomena of fluorescence, fluorescence imaging technologies were developed in order to visualize and study the organic or inorganic substances. In most cases, desired part of the specimen, e.g ions or organelles are specifically labeled with a fluorescent molecule. Those fluorescent molecules are called fluorophores (Calcium green, Lucifer yellow). Those fluorophores are excited by a specific wavelength of light and emit longer wavelengts of light. Most of the modern fluorescence microscopes use epi-luminescence technique, in which both excitation and emission lights will have a common optical path through the objective (fig. B.1) The dichroic mirror reflects the wavelength below its cutoff frequency and transmits those which are above the cutoff. This cutoff frequency should be chosen so that it will reflect all of the excitation wavelength, and transmit most of the emission wavelength. Xenon-arc lamp is used for excitation. (250-1000 nm) The fundamental principle underlying a fluorimeter is the maximization of the collection of the fluorescence emission and trying to minimize the collection of excitation light. This is usually accomplished by selecting a band of excitation wavelength which will not be present in the emission spectrum by the use of filters (interference or combination filters), or a monochromator on the excitation side, and highpass or bandpass filters on the emission side. Typical components of a fluorescence microscope are the light source (Xenon or Mercury arc-discharge lamp), the excitation filter, the dichroic mirror (or dichromatic beamsplitter), and the emission filter. The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen.

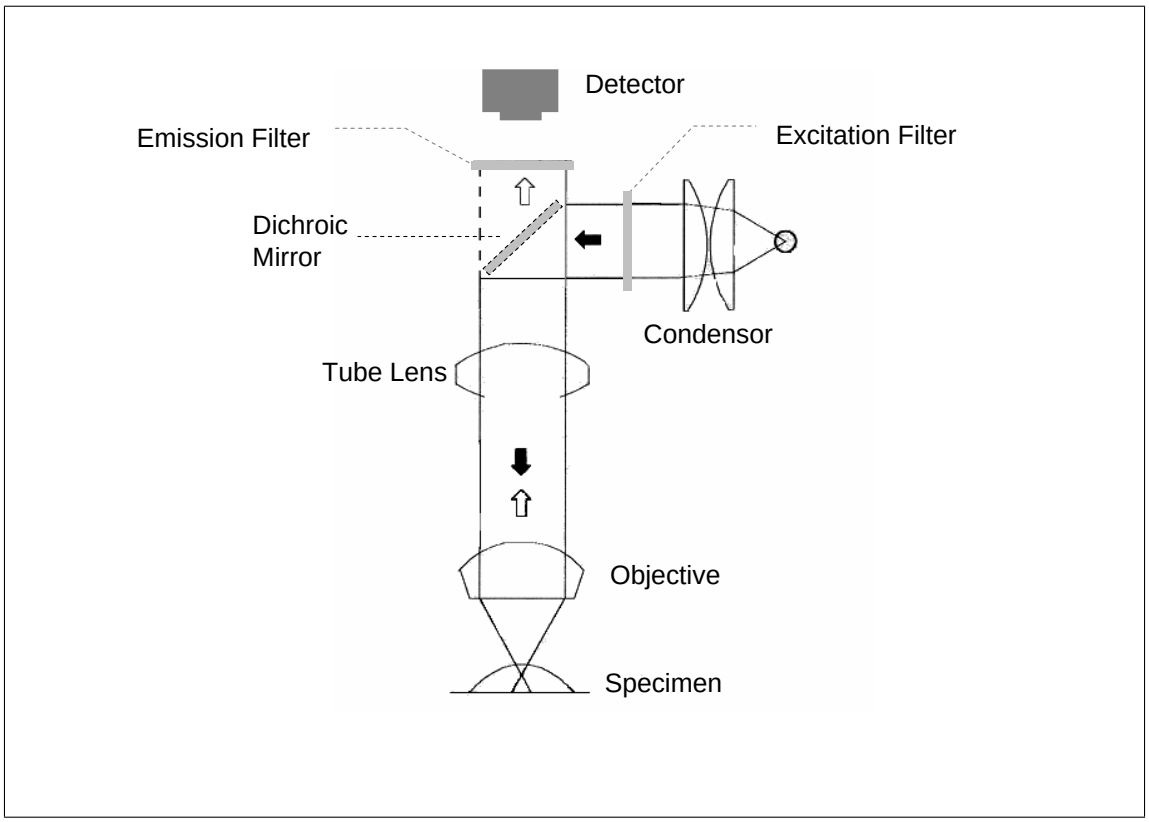

Figure B.1 Epifluorescence microscope and its main components

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