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AN ELECTROPHYSIOLOGICAL INVESTIGATION OF MONOSIALOGANGLIOSIDE IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM

by

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Canada

Running title for the Ph. D. thesis submitted by PETER MIU:

Actions of GM1 in the mammalian central nervous system.

I LIKE TO DEDICATE THIS THESIS TO MY WIFE, Natushka.

".... to all who turn to science, not because they intend to take it up to - write or discuss or teach - but to get answers to straight, simple everyday problems."

Tolstoy, Resurrection.

ABSTRACT

This thesis focuses on the functional role of monosialoganglioside (GM1) in neuronal activity and synaptic transmission of rat hippocampal slices. The slices were placed in an interface recording chamber and constantly superfused with oxygenated saline at 33°. Both extracellular and intracellular (current- and voltage-clamp) recording techniques were used to measure the field potentials and postsynaptic responses respectively. Our findings indicated that GM1 induces a small inward current in CA1 pyramidal neurons, depresses their high voltage activated (HVA) Ca^{2+} currents, and selectively facilitates the excitatory synaptic inputs while reducing the inhibitory ones.

The most probable mechanism underlying the selective enhancement of excitatory inputs by GM1 is an increase in glutamate release, since the amplitudes and frequency of spontaneous miniature postsynaptic responses, recorded either in the presence or absence of presynaptic cell firing, were consistently increased. When L-glutamate was applied iontophoretically in the dendritic region, GM1 transiently potentiated the postsynaptic glutamate currents, thus further indicating a GM1-induced enhancement of glutamatergic transmission. In contrast, both spontaneous and evoked inhibitory postsynaptic responses were suppressed by GM1. This effect is dependent on changes in excitatory inputs to inhibitory interneurons because in the presence of tetrodotoxin and/or kynurenic acid, GM1 did not alter the amplitude of the monosynaptic IPSPs or the frequency of spontaneous miniature IPSCs.

Both the GM1-induced inward current and the reduction of postsynaptic HVA Ca^{2+} currents were antagonised by kynurenic acid, suggesting that these effects might be caused by glutamate receptor activation. By raising intraneuronal Ca^{2+} concentration, the potentiated glutamate release would trigger Ca^{2+}-dependent Ca^{2+} inactivation, and thus explain the reduction in HVA Ca^{2+} currents.

In conclusion, most of the GM1 actions observed in this project can be explained on the basis of a GM1-induced facilitation of excitatory transmission, mediated especially via enhanced glutamate release.

RÉSUMÉ

Les présentes expériences, réalisées sur tranches de cerveau prélevées sur l'hippocampe de rat, ont été consacrées a l'étude du rôle fonctionnel du monoganglioside (GM1) dans l'activité nerveuse et la transmission synaptique. Les tranches de cerveau ont été maintenues in vitro dans une chambre de survie à l'interface entre un flux d'oxygène (O2/CO2, 95%/5%) et une solution saline, à température de 33°. Les techniques d'enregistrement extra- et intracellulaire (à courant constant et en voltage imposé) ont été utilisées pour enregistrer, respectivement, les potentiels de champ et les réponses postsynaptiques.

Nos observations montrent que, dans les neurones pyramidaux CA1, le GM1 induit un petit courant entrant, qu'il déprime les courants calciques déclenchés par les potentiels de membrane élevés du type 'HVA', et qu'il facilite sélectivement les inputs synaptiques excitateurs, tout en réduisant les inputs inhibiteurs. L'augmentation sélective des inputs excitateurs par le GM1 résulte très probablement d'une augmentation de libération du glutamate; en effet, l'amplitude et la fréquence des potentiels postsynaptiques (PPSE) miniatures spontanés sont notablement accrues, que ce soit en présence ou absence de décharges cellulaires présynaptiques. Le GM1 produit aussi un accroissement temporaire des courants postsynaptiques induits par une application iontophorétique de glutamate au niveau dendritique; cette observation renforce donc notre hypothèse selon laquelle le GM1 favorise la transmission glutamatergique. Par ailleurs, les réponses postsynaptiques inhibitrices (PPSIs), qu'elles soient spontanées ou évoquées, sont supprimées par le GM1. Cet effet dépend de modifications dans les inputs excitateurs des interneurones inhibiteurs, car, en présence de tétrodotoxine ou d'acide kynurénique, le GM1 n'influence ni l'amplitude des PPSI monosynaptiques evoqués, ni la fréquence des PPSI miniatures spontanés.

Le fait que l'acide kynurénique contrecarre l'induction d'un courant entrant et la réduction des courants calciques HVA par le GM1, suggère fortement que ces effets résultent d'une activation des récepteurs glutamatergiques. En augmentant la concentration intraneuronale de calcium, une libération accrue de glutamate engendrerait une inactivation calcium-dépendante des courants calciques HVA.

En conclusion, la plupart des effets du GM1 décrits dans notre travail, peuvent être expliqués par l'effet facilitateur du GM1 sur la transmission synaptique excitatrice, modulée principalement via un accroissement de libération du glutamate.

PREFACE

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The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines concerning thesis preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion.

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The work reported in this thesis was carried out by the author in Dr. K. Krnjević's laboratory at McIntyre Medical Sciences building of McGill University.

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LIST OF ABBREVATIONS

Artificial cerebrospinal fluid	ACSF
Tetrodotoxin	TTX
Tetraethylammonium	TEA
Four aminopyridine	4-AP
Calcium	Ca^{2+}
Manganese	Mn^{2+}
Acetylcholine	ACh
γ-amino-butyric acid	GABA
Bicuculline methiodide	BMI
Monosialoganglioside	GM1
Metabotropic glutumate receptor	mGluR
N-Methyl-d-Aspartate	NMDA
Kynurenic acid	KYN
High-voltage-activated calcium currents	HVA-Ca^{2+} currents
Extracellular calcium concentration	$[Ca^{2+}]_o$
Guanosine nucleotide binding protein	G-protein
Adenosine 3',5'-monophosphate	cyclic AMP
Inositol trisphosphate	IP_3
Protein kinase C	PKC
Diacylglycerol	DAG
Phorbol-12, 13-diacetate	PDAc

CHAPTER 1

1.1 INTRODUCTION

The word gangliosides refers to a group of sialic acid-containing glycosphingolipids predominantly found in the plasma membrane of nerve terminals. These compounds contain a hydrophobic portion which interacts with the plasma membrane, and a hydrophilic portion which extends into the extracellular milieu (Fig. 1.1).

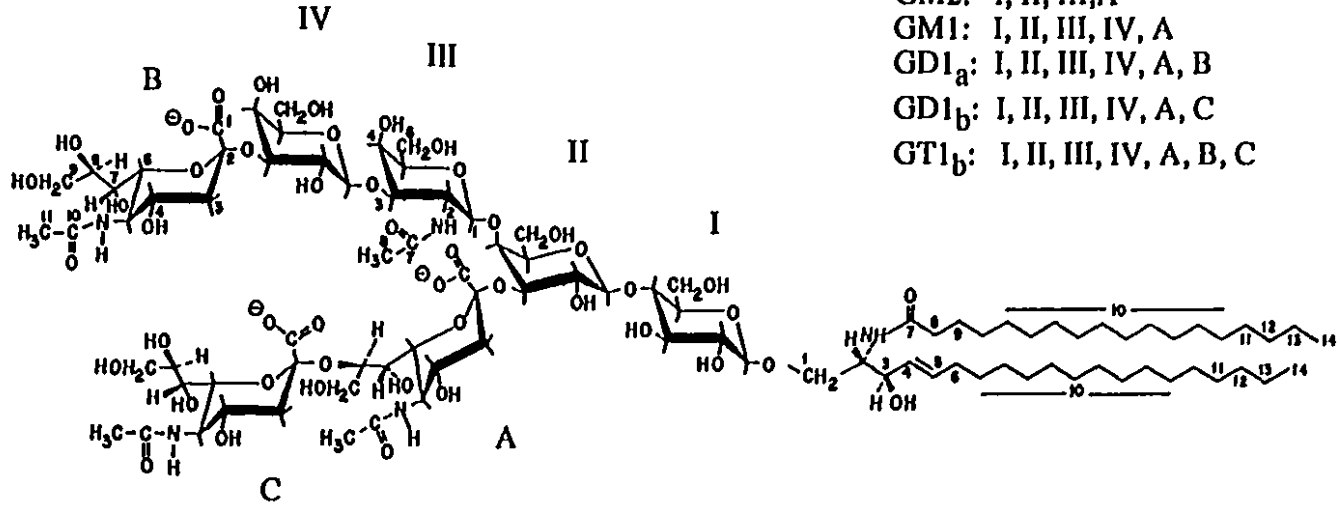
In view of the location of gangliosides and their ability to interact with the extracellular environment, gangliosides have been postulated to exert a powerful influence on synaptic transmission and plasticity, by modulating various ligand-gated receptor channels and second messenger actions. However, the underlying mechanisms for the improvement in the efficacy of synaptic transmission remain elusive. In this context, this thesis examines the functional role of gangliosides, in particular the monosialoganglioside (GM1), in the mammalian central nervous system.

1.2 Historical Perspectives

Sphingolipids were initially isolated from human and bovine brains by a physician scientist named Thudicum in 1884. From these preparations, he extracted not only the phospholipids but also three types of sphingosine-containing lipids which were classified as sphingomyelin, cerebroside, and cerebrosulfatide (Fig. 1.2).

Since the discovery of sphingolipids by Thudicum, further studies on the chemical

GM4: II, A
GM3: I, II, A
GM2: I, II, III,A
GM1: I, II, III, IV, A
$GD1_a$: I, II, III, IV, A, B
$GD1_b$: I, II, III, IV, A, C
$GT1_b$: I, II, III, IV, A, B, C



Oligosaccharide (hydrophilic) moeity

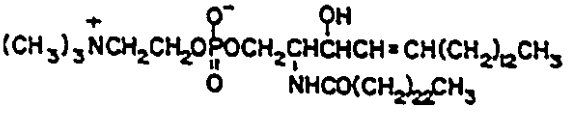
Ceramide (hydrophobic) moeity

Figure 1.1: Parental structure of brain gangliosides.

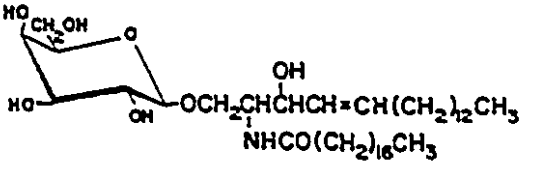
structures of sphingolipids were impeded by the difficulties associated with the extraction and subsequent isolation of these compounds from brain specimens, as well as the indifference among the biochemists towards these lipids! Nonetheless, enthusiasm was promptly renewed within the scientific community following the discovery that several human diseases are associated with disorders of sphingolipid metabolism. These disorders are typically characterized by a genetically linked enzyme deficiency, resulting in massive accumulation of various sphingolipids such as sphingomyelin (Niemann-Pick disease), cerebroside (Krabbe disease), and sialic acid-containing glycosphingolipids such as the gangliosides (Tay-Sachs and Sandoff diseases, as well as GM1 gangliosidosis) (Beaudet, 1991).

Using brain samples obtained from patients with Tay-Sachs disease, Klenk (1941) detected large quantities of gangliosides which were known at the time as 'substance X'. Because Klenk found that the content of 'substance X' was much higher in gray matter than in white matter of the brain, he subsequently named it (in the same paper) Neuraminsäure or ganglioside. In the following year, he successfully isolated and characterized the ganglioside from bovine brain (Klenk, 1942; Klenk and Rennkamp, 1942). Until the early fifties, ganglioside was considered to be a single compound; however, within the last four decades, significant progress was achieved in elucidating several variants of the chemical structure and the biosynthetic pathways of brain gangliosides.

Sphingomyelin



Cerebroside



Cerebrosulfatide

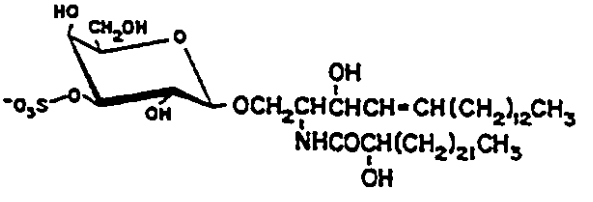


Figure 1.2: Chemical structures of sphingosine-containing lipids.

1.3 Chemical Structure of Brain Gangliosides

Studies on brain ganglioside by Klenk (1941 & 1942; Klenk and Rennkamp, 1942) showed that this compound contains a sialic acid residue attached to an oligosaccharide chain. In 1963, detailed structural compositions of the four major brain gangliosides in normal mammalian brain were elucidated and subsequently named GM1, $GD1_a$, $GD1_b$, and $GT1_b$ (Kuhn and Wiegandt, 1963*a* & 1963*b*; Svennerholm, 1963). These make up the ganglio-series. In the ensuing years, new classes of gangliosides were discovered in bovine erythrocytes and human spleen - known as the lacto-series - (Kuhn and Wiegandt, 1964; Wiegandt and Schulze, 1969; Li et al.., 1973; Wiegandt, 1973 & 1974), and in bovine liver and pig testes - known as the fuco-series (Wiegandt, 1973; Suzuki et al., 1975). I shall focus exclusively on brain gangliosides belonging to the ganglio-series. These brain gangliosides contains the hexosamine, stearic acid, and N-acetylneuraminic acid, which are not found in any other glycolipids (Klenk, 1959; Klenk and Padberg, 1962).

Typically all brain gangliosides have a similar parental structure (Fig. 1.1); that is, they are composed of a hydrophobic (or ceramide) moiety and a hydrophilic (or oligosaccharide) moiety. The ceramide portion contains sphingosine and a fatty acid (mainly stearic) chain, linked by an amide bond; whereas the oligosaccharide portion contains glucose, galactose, N-acetylgalactosamine (linked by glycosidic bonds), and sialic acid (or N-acetylneuraminic acid; NANA) coupled to galactose or another NANA. The distinguishing feature of brain gangliosides is the extensive sialylation which is not found

in gangliosides extracted from non-neuronal tissues (Handa and Burton, 1969).

Hence, variations of this parental structure by changes in the position of NANA coupling lead to the formation of the four major gangliosides mentioned earlier, and five other minor brain gangliosides (more below). The standardized nomenclature of these brain gangliosides in recent literature has been abbreviated from the generic term by Svennerholm (1970), in order to hasten oral or written communication. Therefore, the first letter in, for example, $GD1_b$ denotes gangliosides belonging to the ganglio-series; the second letter denotes the number of sialic acids attached, hence M, D, T, and Q indicates mono-, di-, tri-, and quatro-sialic acid residues respectively; the number refers to the carbon atoms of the hexopyranoside residues; and lastly the subscript letter refers to the carbon to which sialic acid is attached (Sillerud et al., 1978 & 1982).

1.4 Metabolism of Brain Gangliosides

1.4.1 Biosynthesis

The systematic elucidation of the biosynthetic pathways of mammalian brain gangliosides was pioneered by Basu and colleagues in the late sixties (Basu et al., 1965 & 1968*a*, *b*). Embryonic chicken brain was selected on the basis that the ganglioside content increases much more rapidly in chicken brain than in rat brain (Carrigan and Chargaff, 1963; Rosenberg and Stern, 1966; Basu et al., 1968*a*).

Basu et al. (1968*b*) showed that gangliosides were synthesized by a step-wise

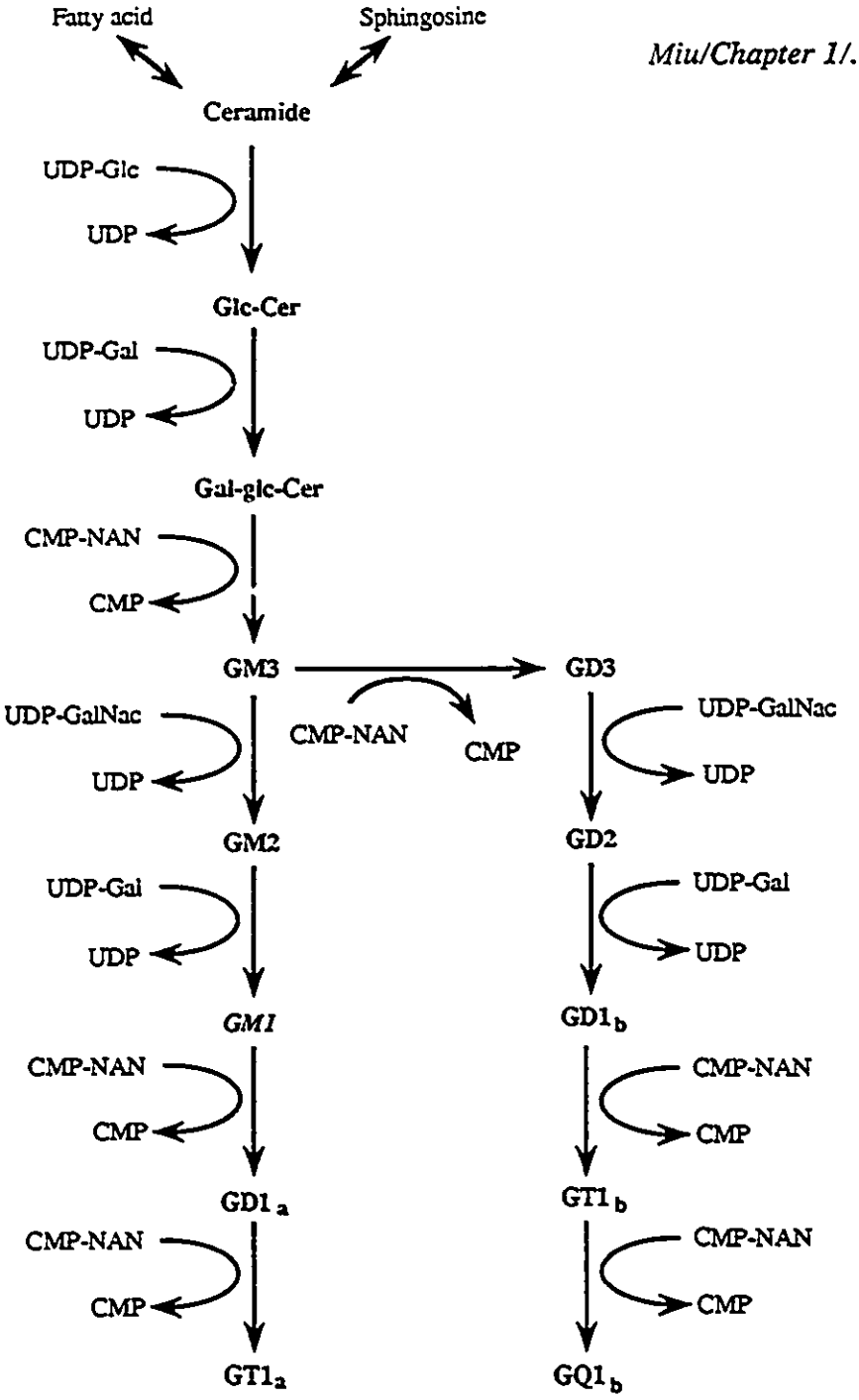


Figure 1.3: Synthetic pathways of both major and minor brain gangliosides.

incorporation of monosaccharides and NANA residues to the appropriate acceptors. Figure 1.3 illustrates the synthetic pathways of brain gangliosides proposed by these investigators. As shown in this figure, two pathways were identified with GM3 as the pivotal point for the subsequent production of major brain gangliosides such as GM2, GM1, $GD1_a$, and $GT1_a$, and minor brain gangliosides such as GD3, GD2, $GD1_b$, $GT1_b$, and $GQ1_b$. The distribution profile of human and rat brain gangliosides indicates that GM1 is one of the four major gangliosides expressed in high concentrations (Table 1.1).

Two principal chemical reactions are involved in these biosynthetic pathways: 1) incorporation and elongation of monosaccharides, and 2) addition of NANA residues to the glycosphingolipids. Even if glycosyltransferases and sialosyltransferases are the two major classes of enzymes, each step in the synthetic pathways is catalysed by a specific enzyme.

For example, the first few steps in the synthesis of GM3 involve a sequential transfer of uridine-5'-diphosphate-glucose(UDP-glc) and uridine-5'-diphosphate-galactose (UDP-gal) to ceramide; and each step is catalysed by a specific glycosyltransferase for glucose and galactose respectively (Basu et al, 1968*a*).

1. Ceramide + UDP-glc → glc-ceramide + UDP; and
2. Glc-ceramide + UDP-gal → gal-glc-ceramide + UDP.

In a reaction catalysed by a specific sialosyltransferase (Basu et al., 1968*b*), cytidine-5'-monophosphate-N-acetylneuraminic acid (CMP-NANA) is then added to this

Table 1: Ganglioside content in brain tissues[a]

Tissue	Lipid-NANA[b]	GM2[c]	GM1	$GD1_a$	$GD1_b$	GT1	GQ1
Human cerebral cortex	1002	1.3	11.3	22.4	28.3	29.9	5.9
Human white matter	156	1.0	9.3	14.0	31.4	38.2	6.4
Rat brain	1047	1.2	13.0	32.1	20.3	27.3	6.0

[a]Modified from Svennerholm, 1970.
[b]Lipid-NANA (N-acetyl-neuraminic acid) expressed as μg/g wet weight.
[c]Distribution of NANA in %.

disaccharide-ceramide compound to yield the end product GM3.

3. Gal-glc-ceramide + CMP-NANA → Gal-glc-ceramide + CMP.
   |
   NANA

There are three different sialosyltransferases which specifically catalyse three different reactions. These reactions include a) catalysation of CMP-NANA to the terminal galactose of the diglycosylceramide, mentioned above, b) catalysation of CMP-NANA to the terminal galactose of the N-tetraglycosylceramide, and lastly c) catalysation of CMP-NANA to the other sialic acid residue attached to the oligosaccharide glycosphingolipids (Basu et al., 1968*b*).

Hence, the production of different gangliosides within the synthetic pathways depends exclusively on its precursor, since the product of each reaction also serves as the substrate for the next transferase (Svennerholm, 1970).

1.4.2 Degradation

Like the step-wise biosynthesis of brain gangliosides, degradation also follows a sequential removal of the monosaccharides and sialic acid residues from the gangliosides by a family of lysosomal exoglycosidases (Fig. 1.4). These enzymes include neuraminidase, β-galactosidase, β-glucosidase, and β-N-acetylhexosaminidase (Gatt, 1970; Brunngraber, 1979; Rosenberg, 1980; Ledeen, 1983; Tettamanti, 1983).

All gangliosides containing multiple sialic acid residues are ultimately broken down to GM1 or GM2 by neuraminidase (Carubelli et al., 1962; Morgan and Laurell, 1963; Tettamanti et al., 1975; Tettamanti, 1983). The sialic acid residue in GM1 or GM2, however, is resistant to neuraminidase because of steric hindrance caused by the presence of terminal galactose and galactosamine (Kuhn and Wiegandt, 1963*a*; Leibovitz and Gatt, 1968; Ohman et al, 1970; Drzeniek, 1973; Rosenberg, 1980; Ledeen, 1983). Therefore complete hydrolysis of GM1 requires a step-wise removal of both terminal monosaccharides by specific glycosidases, resulting in the formation of ceramide (Korey and Stan, 1963; Svennerholm, 1970). Further degradation of ceramide by ceramidase yields the fatty acid and sphingosine. Recently, increasing evidence suggests that sphingosine, derived from ceramide hydrolysis, acts as an inhibitor of the two major Ca^{2+}-dependent second messenger systems, namely protein kinase C and Ca^{2+}/calmodulin dependent kinase (Hannun et al., 1986; Kreutter et al., 1987; Jefferson and Schulman, 1988; Hannun and Bell, 1989; Riboni et al., 1992).

1.4.3 Localization of synthetic and catabolic enzymes

1.4.3.i Synthetic enzymes

Early preparations using 'pinched off' nerve endings (synaptosomes) suggested that gangliosides were synthesized in the plasma membrane of nerve terminals (Den et al., 1970 & 1975; Di Cesare and Dain, 1971 & 1972). However, later experiments showed that most of these membrane preparations were contaminated by membranes of the Golgi

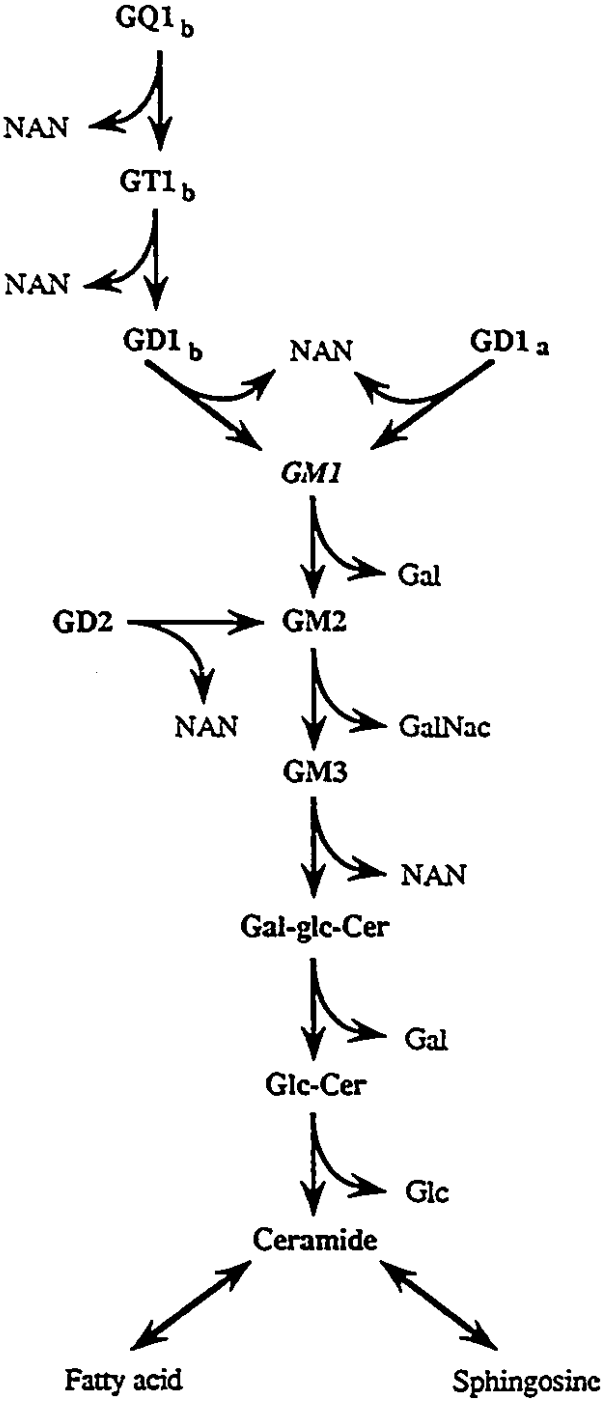


Figure 1.4: Degradation pathways of brain gangliosides.

apparatus and/or endoplasmic reticulum (Reith et al., 1972; Raghupathy et al., 1972; Landa et al., 1979).

Indeed, there is convincing evidence that in non-neuronal preparations, most of the glycosyltransferases are located in the Golgi apparatus (Keenan, 1974; Keenan et al., 1974; Wilkinson et al., 1976). Subsequent studies have characterized and extracted all the glycosyl- and sialosyl-transferases responsible for the complete synthesis of gangliosides from rat liver Golgi apparatus (Wilkinson, 1976; Richardson et al., 1977; Eppler et al., 1980; Senn et al., 1981 & 1983; Kaplan and Hechtman, 1983; Yusuf et al., 1983).

On the basis of these findings, it is postulated that gangliosides are synthesized in the Golgi apparatus and transported in the Golgi vesicles, by orthograde axonal flow, towards the plasma membrane, as illustrated in figure 1.5. The newly synthesized gangliosides are then incorporated, into the plasma membrane by membrane fusion, with the oligosaccharide chains facing the extracellular milieu (Tettamanti, 1983).

A similar working model for nerve cells has received support from recent studies showing that gangliosides are synthesized in the neuronal perikaryon and later transported into the nerve terminals (Forman and Ledeen, 1972; Holm, 1972; Rösner et al., 1973; Rahmann and Breer, 1975; Rösner, 1975; Landa et al., 1979).

1.4.3.ii Catabolic enzymes

Most of the catabolic enzymes are located exclusively in the lysosomes (Ohman et al., 1970; Ragahavan et al., 1972; Abe et al., 1979; Gatt, 1979). The only

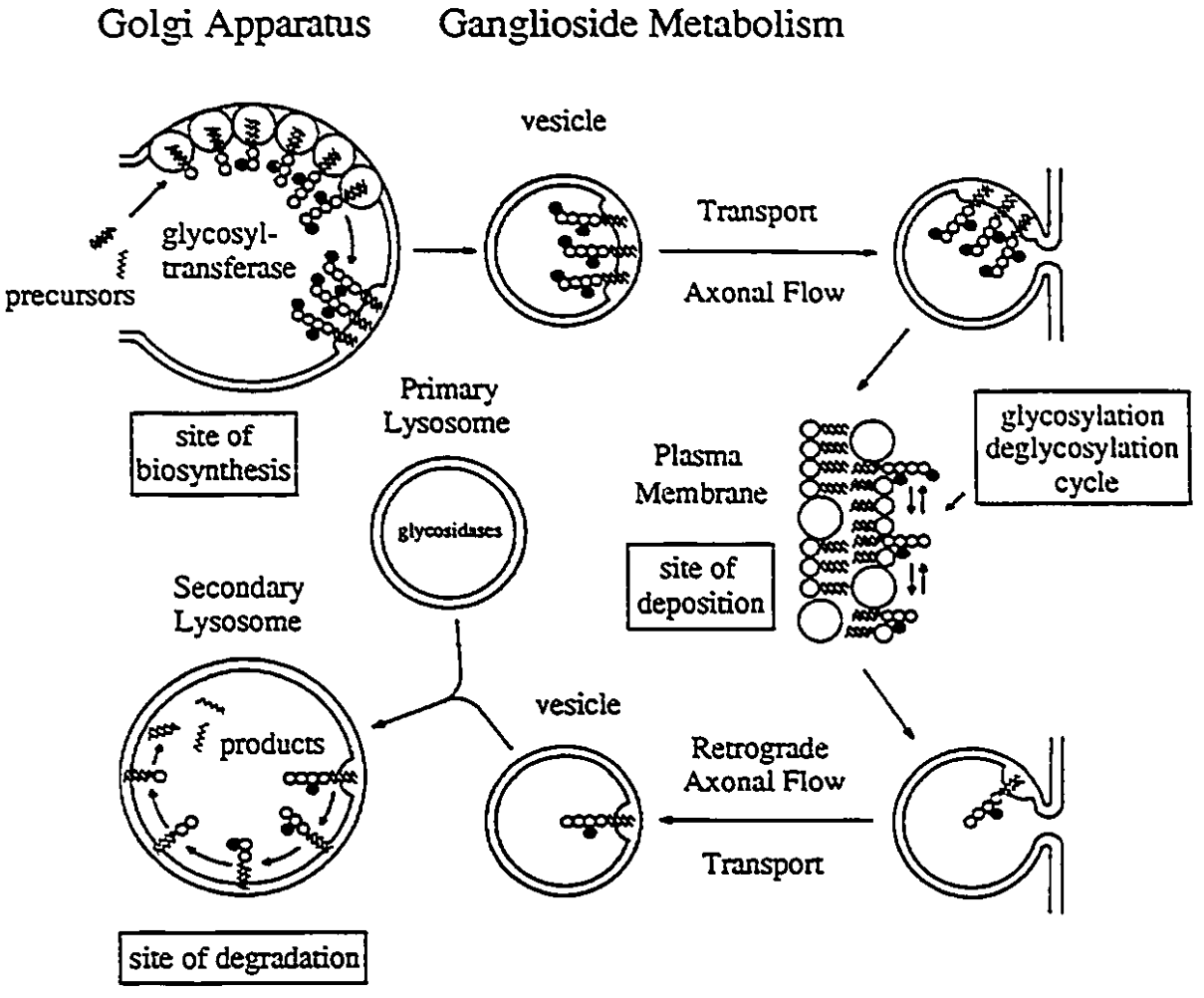


Figure 1.5: Working model of ganglioside metabolism.

exception is neuraminidase, which is located not only in the lysosomal fractions but also in synaptosomal plasma membrane (Schengrund and Rosenberg, 1970; Tettamanti et al., 1972; Dain and Ng, 1979; Tettamanti et al., 1979).

Therefore, most of the de-sialylation occurs within the plasma membrane, prior to or during membrane internalization. Complete hydrolysis of gangliosides begins when the internalized membrane vesicle fuses with primary lysosome, containing most of the glycosidases mentioned earlier, to form the secondary lysosome (Tettamanti, 1983).

1.4.4 Metabolic disorders

According to these findings, the route for ganglioside degradation follows a series of hydrolytic reactions. Each step within the catabolic pathways is catalysed by a specific glycosidase or neuraminidase. Hence these enzymes play a paramount role in maintaining proper concentrations of different gangliosides within the plasma membrane, and thus prevent abnormal accumulation of ganglioside within the plasma membrane, which could result in functional impairment of the whole organism.

Table 1.2 shows some examples of metabolic disorders related to sphingolipids. In all cases, a genetically-linked deficiency in one enzyme results in excessive accumulation of the substrate for the missing enzyme. Furthermore, a common manifestation of these diseases is a neurologic impairment, characterized by mental retardation (Beaudet, 1991).

The neurological impairment induced by excess accumulation of a ganglioside is,

Table 2: Inherited disorders of sphingolipid metabolism[a]

Enzyme deficiency	Disorder	Accumulation of
β-Galactosidase	GM1 gangliosidosis	GM1
Hexosaminidase A	Tay-Sachs	GM2
Hexosaminidase A, B	Sandhoff	GM2
β-Glucosidase	Gaucher	Glucosylceramide

[a]Modified from Beaudet, 1991.

perhaps, not unexpected in view of the fact that gangliosides are found predominantly in the brain (Table 1.3). Moreover, gangliosides have a distribution profile similar to that of γ-aminobutyric acid (GABA) in various brain regions suggesting that gangliosides may modulate synaptic transmission (Roberts, 1962; Lovell and Elliott, 1963; Lowden and Wolfe, 1964; Derry and Wolfe, 1967). This hypothesis is further supported by the findings that among different subcellular fractions of nerve cell preparations, high concentrations of gangliosides are consistently found in the synaptic plasma membranes (Table 1.4).

1.5 Functional Role of Gangliosides

1.5.1 Gangliosides as surface membrane receptors

Before the late sixties, centrally acting convulsive agents such as tetanus toxin, strychnine, brucine, protamine, and thebaine were known to reduce inhibitory synaptic transmission in the spinal cord and cerebral cortex (Sherrington, 1906; Bradley et al., 1953; Brooks et al., 1957; Curtis, 1959; Wilson et al., 1960; Brooks and Asanuma, 1962; McIlwain, 1963; Eccles, 1964). However, the mechanisms underlying the attenuation of inhibitory synaptic transmission by these agents were unclear, since the roles of GABA and glycine in the nervous system, at the time, were still unresolved (Eccles, 1964).

Using soluble extracts of brain gangliosides, van Heyningen and collaborators showed that pre-incubation with brain ganglioside extracts blocks the physiological effects

Table 3: Ganglioside content of human tissues[a]

Tissue	Concentration[b]
Brain gray matter	2850-3530
Brain white matter	900-1570
Spinal cord gray matter	751
Spinal cord white matter	430
Retina	366
Sciatic nerve	259
Adrenal medulla	407-757
Muscle	52
Liver	214
Blood plasma	11.3
CSF (lumbar)	0.8
CSF (ventricular)	0.3

[a]Modified from Ledeen, 1983.
[b]Tissue concentrations are expressed as nmoles of lipid-bound sialic acid per g. fresh weight; plasma and CSF are expressed as nmoles lipid-bound sialic acid per ml.

induced by these convulsive agents, and that only brain gangliosides containing sialic acid residues and hexosamine group can bind with high specificity to these compounds (van Heyningen, 1959*a*, *b*, & *c*; van Heyningen and Miller, 1961; van Heyningen, 1963). Subsequent isolation of cholera toxin, the enterotoxin from *Vibrio cholera* (De, 1959; Craig, 1965 & 1966; Finkelstein, 1969; Finkelstein and LoSpalluto, 1972), further characterized GM1 as the most effective brain ganglioside in neutralizing the toxic action of cholera toxin prior to membrane binding (van Heyningen et al., 1971; King and van Heyningen, 1973; Cuatrecasas, 1973*a* & *b*; Holmgren et al., 1973).

Based on these observations, the concept that gangliosides function as specific surface membrane receptors for these compounds seemed firmly established. Moreover, this idea was extrapolated further to include other biologically active agents such as serotonin (Wooley and Gommi, 1964 & 1965), opiates (McLawhon et al., 1981), and thyrotropin (Aloj et al., 1977; Kohn et al., 1979). However, subsequent receptor characterization for some of these compounds did not confirm this hypothesis (Becker et al., 1981; Lacetti et al., 1983; Fuller, 1984); consequently, the role of gangliosides in synaptic transmission remains unclear.

1.5.2 Formation of Ca^{2+}-ganglioside complexes

One of the chemical constituents which contributes to the hydrophilic property of brain gangliosides is sialic acid, or N-acetylneuraminic acid (NANA). Owing to its low pK_a (2.6), sialic acid exists mostly in the negatively charged form in physiological saline

Table 4: Gangliosides of neuronal sub-cellular fractions[a]

Sub-cellular fraction	Concentration[b]
Synaptic plasma membrane	
Rat	24-146
Calf	90
Guinea pig	55
Axolemma	
Rat	78
Bovine	49
Human	45
Synaptosomes	
Ox	53
Rabbit	39-45
Rat	24-35
Guinea pig	27
Calf	26
Human	23
Synaptic vesicles	
Rat	9.4-16
Soluble	
Rat synaptosomes	1.1-4.8
Rat neurons and glia	4.3
Axons	
Ox[c]	1.2

[a]Modified from Ledeen, 1983.
[b]Expressed as nmoles of lipid-bound sialic acid per mg protein.
[c]Primarily axoplasm with little or no axolemma.

(Eylar et al., 1962; Jaques et al., 1977). Furthermore, since gangliosides are asymmetrically distributed in the outer leaflet of the plasma membrane, numerous investigators have speculated that brain gangliosides may modulate synaptic transmission through their ability to form Ca^{2+}-ganglioside complexes on the surface of the plasma membrane (Yamakawa and Nagai, 1978; Svennerholm, 1980; Veh and Sander, 1981; Rahmann et al., 1982; Rahmann, 1983; Thomas and Brewer, 1990).

This hypothesis is supported by the observations that sialic acid forms a stable complex with Ca^{2+} at pH 7.0 in low (millimolar range) ionic strength solution (Behr and Lehn, 1972 & 1973; Hutson, 1977), and that the formation of Ca^{2+}-ganglioside complex is highly dependent on the number of sialic acid residues attached to the oligosaccharide chain (Hayashi and Katagiri, 1974; Maggio et al., 1980). However, Maggio and company (1977) showed that gangliosides containing more than one sialic acid residues manifest some structural constraints in the binding site for Ca^{2+}.

When ganglioside micelles are suspended in aqueous solution, the binding affinity of gangliosides to Ca^{2+} is estimated to be in the range of 10^6 to 10^4 M^{-1} (Behr and Lehn, 1973; Probst et al., 1979). In contrast, a much lower intrinsic association constant for binding of Ca^{2+} to gangliosides (0-100 M^{-1}) was reported by McDaniel and McLaughlin (1985). These investigators consequently considered it unlikely that biological membranes use gangliosides as receptors for calcium. Regardless, the binding affinity is substantially reduced when these Ca^{2+}-ganglioside complexes are exposed to high (decimolar range) ionic strength monovalent salt (KCl) solution (Carter and Kanfer, 1973;

Hayashi and Katagiri, 1974; Felgner et al., 1982; McDaniel and McLaughlin, 1985) or Ca^{2+} (Probst et al., 1979).

1.5.2.i Surface charges and channel function

Indeed, sialic acid residues can contribute significantly to the negative charges at or near the surface of a channel (Miller et al., 1983; James and Agnew, 1987). Furthermore reductions in the sialic acid content within the plasma membrane by neuraminidase or sialidase have been shown to influence the subconductance states of the *Electrophorus* sodium channel (Levinson et al., 1990). Other channel conductances which are affected by surface charges include K^+- (Bell and Miller, 1984), Ca^{2+}-dependent K^+- (Moczydlowski et al., 1985) and L-type Ca^{2+}-channels (Coronado and Affolter, 1986).

The importance of surface charge on channel function has been described by two models: the Gouy-Chapman theory of the diffuse double layer (Gouy, 1910; Chapman, 1913), and the Debye-Hückel theory of ionic solutions (Debye and Hückel, 1923).

The Gouy-Chapman model predicts that the surface charge near a channel is uniformly distributed over a planar surface of infinite area. Therefore for an arbitrary electrolyte solution, the relation among a) the valence of ion species (z_s) at bulk concentration c_s and with dielectric constant ε, b) the surface potential (ψ_o), and c) the charge density (σ) can be described by the Grahame (1947) equation,

$$\sigma^2 = 2\varepsilon\varepsilon_o RT \sum_s c_s \left[\exp\left(\frac{-z_s F \psi_o}{RT} \right) - 1 \right] \qquad (1)$$

where the sum is taken over all ions, and ε_o is the electronic charge (Hille, 1984).

The Debye-Hückel theory of activity coefficients for dilute solutions proposes that the surface charge of single ions is distributed over a conducting, impenetrable sphere of radius a and valence z_s. The potential (ψ_o) at the surface of the sphere of radius a is predicted by (Koryta and Dvorak, 1987)

$$\psi_o = \frac{z_s * e}{\left[4\varepsilon\varepsilon_o a\left(\frac{1+a}{L_D}\right)\right]} \qquad (2)$$

where e and L_D represent respectively the elementary charge and the Debye length which is a function of the ionic strength ($\sum cz^2$) and charge defined as,

$$L_D = \sqrt{\frac{\left(\frac{\varepsilon\varepsilon_o RT}{F^2}\right)}{\sum cz^2/2}} \qquad (3)$$

The Debye length provides an estimate for the exponential radius of charge decay with distance from a point source (Hille, 1984). Moreover, the term z^2 also shows that divalent ions will be much more effective in shielding the surface charges than monovalent ions. Therefore both models predict that the magnitude of ψ_o is reduced with increasing ionic strength.

However, these models were based on a simplified membrane system where the

surface charges are assumed to be spread evenly over the entire membrane surface. This assumption is obviously false since not all membranes contain the same number of charged groups interacting with the extracellular milieu (for a detailed review see Hille, 1984). Nevertheless, for the purpose of this Introduction, it is sufficient to say that the negative surface charge would increase the relative concentration of cations, in particular Ca^{2+}, near a channel entrance (Prod'hom et al., 1989; Dani, 1986; Green et al., 1987; Cai and Jordan, 1990).

In view of these observations, it is postulated that upon nerve terminal depolarization, the K^+ concentration in the vicinity of Ca^{2+}-ganglioside complexes increases thereby changing the ionic strength in the synaptic cleft. This effect would reduce the ganglioside-binding affinity for Ca^{2+}, and thus liberate bound Ca^{2+} and this makes more Ca^{2+} available for the transmitter release process (Rahmann et al., 1976 & 1982; Thomas and Brewer, 1990). Another scheme was proposed by Veh (1986) for the neuromuscular junction, where released acetylcholine (ACh) is hydrolysed to choline and acetic acid. Formation of acetic acid would consequently reduce the ionization of gangliosides present in the synaptic cleft thus causing them to release Ca^{2+}.

1.6 Ca^{2+} and Synaptic Transmission

The term synapse, which is derived from the Greek word *σύνάπτω*, was coined by Sherrington in 1897 to describe the nexus (or separation) between two neurons in regions

of close juxta-position. Furthermore, he stated that the delayed response observed in the reflex-arc is mainly caused by the presence of many synapses through which impulses are conveyed to the recipient neuron (Sherrington, 1906).

Sherrington's concept of the synapse was based on the histological observations of neuroanatomists such as Cajal (1910). The mechanism of synaptic transmission, whether electrical or chemical was classified much later when Dale and his collaborators demonstrated that impulses in the form of action potentials do not stimulate muscle fibres, but rather through the release of chemical substances from nerve endings (Dale et al., 1936; Brown et al., 1936). After identifying acetylcholine (ACh) as the chemical substance being released from the nerve terminals, the following sequence was postulated as the process of synaptic transmission (Dale, 1935 & 1937),

$$N \underset{I}{---} ACh \underset{II}{---} M \qquad (4)$$

where N and M represent nerve impulse and muscle fibres respectively. However, the mechanisms mediating I (i.e. release) and II (i.e. ligand-receptor interaction) in the above steps were still unknown.

The first evidence suggesting that Ca^{2+} is involved in regulating transmitter release from nerve endings was from the classical study of Harvey and MacIntosh (1940); later much elaborated by Katz and Miledi, in the late sixties, in a series of experiments on the frog neuromuscular junction, where they demonstrated that both the presence of extracellular Ca^{2+} and Ca^{2+} influx via Ca^{2+} channels at a crucial time play a paramount

role in regulating transmitter release (Katz and Miledi, 1967*a*, 1967*b*, & 1967*c*). Similar results were also obtained from presynaptic terminals of the giant synapse of the squid (Bloedel et al., 1966; Katz and Miledi, 1966 & 1967*d*; Kusano et al., 1967). Activation of Ca^{2+} channels allows the build-up of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$), which subsequently triggers exocytosis (Miledi, 1973; Llinás and Nicholson, 1975). These observations formed the corner stone of our present understanding of the mechanisms underlying transmitter release.

Although $[Ca^{2+}]_i$ ($\sim 0.1\ \mu M$) is stringently regulated by various intracellular Ca^{2+}-buffering systems, transient accumulation of cytosolic free Ca^{2+} during an action potential is estimated to reach 1 or even 10 μM (McGraw et al., 1982). Over the last few decades, an overwhelming amount of information has been collected from studies on Ca^{2+} channels responsible for the action potential-dependent Ca^{2+} influx; these Ca^{2+} channels are known as the voltage-dependent Ca^{2+} channels.

1.6.1 Voltage-dependent Ca^{2+} channels

Voltage-dependent Ca^{2+} channels are highly selective for Ca^{2+} (Almers et al., 1986; Kostyuk and Mironov, 1986; Tsien et al., 1987). However, there is a great heterogeneity among various Ca^{2+} channels owing to their different channel activation/inactivation kinetics and conductances.

On the basis of the differences in their biophysical properties and pharmacological profiles, these voltage-dependent Ca^{2+} channels are subsequently subdivided into four

distinct types, and arbitrarily designated as T- (transient), L- (long-lasting), N- (neither T nor L; Nowycky et al., 1985), and the more recently discovered P-types (Purkinje; Llinás et al., 1989). The three most studied channels to date, however, are the T-, L-, and N-types; and there is evidence showing that hippocampal neurons do posses all three types (Brown et al., 1990; Fisher et al., 1990). Both biophysical properties and pharmacological profiles of these four voltage-dependent Ca^{2+} channels will be briefly reviewed below.

1.6.1.i T-type (transient) channels were first described by Carbone and Lux (1984) as the 'low-threshold-activated' Ca^{2+} channels since they can be activated by small depolarizations from negative holding potentials ranging from -100 to -60 mV. Channel activation, however, is followed by fast inactivation, and complete inactivation occurs at holding potentials more positive than -50 mV. Due to the transient channel opening time, T-type channels are typically characterized by a low unitary conductance in comparison to the other types of Ca^{2+} channels. In adult hippocampal neurons, T-type channels have a single-channel slope conductance of approximately 8 pS (Fisher et al., 1990)[1]. It is postulated that these channels contribute to pacemaker activities and repetitive firing in heart and neurons rather than extensive $[Ca^{2+}]_i$ homeostasis (Meldolesi and Pozzan, 1987; Bean, 1989; Hess, 1990). Pharmacological studies showed that the T-type is sensitive to

[1] The single-channel slope conductances for the three voltage-dependent Ca^{2+} channels vary slightly from preparation to preparation. Therefore I have reported only one set of values measured by Fisher et al (1990) from pyramidal neurons of rat hippocampal slices.

inorganic blockers such as Co^{2+} and Ni^{2+}, but insensitive to organic blockers such as dihydropyridines (DHP; Nowycky et al., 1985; Reuter et al., 1985).

1.6.1.ii L-type (long-lasting) channels are best known for their high conductance and prolonged channel opening time. In adult hippocampal neurons, L-type channels have the largest single-channel slope conductance (~25 pS; Fisher et al., 1990). These channels are known as the 'high voltage-activated' Ca^{2+} channels since they activate at holding potentials more positive than ~-30 mV, and reach a maximum at ~+10 mV. Inactivation is very slow, and in most cases governed by various factors such as $[Ca^{2+}]_i$ (Hagiwara, 1981; Fenwick et al., 1982; Eckert and Chad, 1984), neurotransmitters (Gross and MacDonald, 1987; Lipscome et al., 1989; Wanke et al., 1987), and second messengers (Gross and MacDonald, 1989*a* & *b*; Doerner and Alger, 1988; Doerner et al., 1988).

Hence, due to their large conductance and prolonged channel opening time, L-type channels have been postulated to participate in $[Ca^{2+}]_i$ homeostasis and possibly Ca^{2+}-dependent transmitter release. Organic compounds such as verapamil, diltiazem, or DHP are blockers of L-type channels; nevertheless, the nature of DHP modulation is still very controversial (for a detailed review see Miller, 1987).

1.6.1.iii N-type ("neither T nor L") channels were first described by Nowycky and collaborators, in sensory neurons of the chick dorsal root ganglion (1985); and so far,

they have been found exclusively in neurons (Nowycky et al., 1985; Hess et al., 1986; Scott and Dolphin, 1986). These channels, which are also classified as high voltage-activated channels, typically become active at ~-25 mV, and reach a half-maximum at ~0 mV. Channel inactivation is voltage-dependent; inactivation time constants (τ) ranging from ~20 ms to ~1 s have been reported (Hirning et al., 1988; Lemos and Nowycky, 1989). As in the case of L-type channels, inactivation is also modulated by a variety of neurotransmitters (Gross and MacDonald, 1987; Lipscome et al., 1989; Wanke et al., 1987) and second messengers (Gross and MacDonald, 1989*a* & *b*; Doerner and Alger, 1988; Doerner et al., 1988). In adult hippocampal neurons, N-type channels have a single-channel slope conductance (~ 14 pS) intermediate between the T- and L-type (Fisher et al., 1990).

Since N-type channels are also activated at more depolarized holding potentials and these channels may or may not show inactivation, it is difficult to differentiate them from the L-type channels according to biophysical criteria. Nevertheless, these channels are distinguished from the L-type by the finding that they are only sensitive to the Ca^{2+} channel antagonist ω-conotoxin but not DHP. There is also evidence that these channels regulate transmitter release (Perney et al., 1986; Rane et al., 1987; Miller, 1987; Hirning et al., 1988; Agopyan et al., 1992).

1.6.1.iv P-type channels were first identified in the cerebellar Purkinje cells, and described by Llinás and company (1989) as another class of high-voltage activated Ca^{2+}

channels, which regulate transmitter release. The activation/inactivation kinetics of P-type channels are similar to those of L/N types; however these channels are insensitive to both ω-conotoxin and DHP. The most effective antagonist for P-type channels is the funnel-web spider toxin (FTX).

1.7 Second Messengers and Intracellular Ca^{2+} Concentration

More recently, increasing evidence suggests that cytosolic second messengers also play a vital role in modulating intracellular Ca^{2+} concentration. In this regard, receptors coupled to G-proteins have gained a tremendous amount of attention in the last few decades. It has been shown that G-protein activation not only directly modulates ion channels, in particular L-type Ca^{2+} channels (Dolphin, 1990; Schultz et al., 1990), but also triggers a second messenger cascade resulting in a wide range of cellular responses.

1.7.1 G-proteins

G-proteins are heterotrimeric (with subunits designated as α, β, and γ) guanine nucleotide-binding proteins attached to the inner face of the plasma membrane (Brown and Birnbaumer, 1990). Thus far, five distinct groups of G-proteins have been classified according to the functional role of their α-subunits: 1) G_s which activates adenylate cyclase, 2) G_t which is coupled to phosphodiesterase, 3) G_i which inhibits adenylate cyclase, 4) G_o whose function is still unknown, but it is predominantly found in

the hippocampus and co-localized with protein kinase C (Worley et al., 1986), and 5) G_p which couples to phospholipase C (Gilman, 1987; Graziano and Gilman, 1987).

Activation and inactivation of G-proteins are dependent on the hydrolysis of guanosine 5'-triphosphate (GTP) to guanosine 5'-diphosphate (GDP) and formation of the heterotrimeric subunits (α, β, and γ) respectively. That is, in the inactive state, GDP is bound to the catalytic site of GTPase, which is localized in the α-subunit. Interaction of an agonist with its receptor-coupled to G-proteins induces conformational changes, resulting in the release of GDP from the α-subunit. Subsequent binding and hydrolysis of GTP causes the α-subunit to dissociate from the $\beta\gamma$-subunits, thus activating G-proteins. Inactivation of G-proteins occurs when the GDP-α-subunit complex re-associates with the $\beta\gamma$-subunits (Dunlap et al., 1987; Ross, 1989; Sato, 1989; Birnbaumer et al., 1990).

While in the dissociated state, the α-subunit has been shown to directly open K^+-channels coupled to the muscarinic receptors (M_2) of guinea pig atrial cells (Yatani et al., 1988*a* & *b*). In contrast, other investigators have shown that the $\beta\gamma$-subunits are involved in the opening of K^+-channels (Logothetis et al., 1987; Neer and Clapham, 1988; Kim et al., 1989). Thus far, the regulatory roles of α- and $\beta\gamma$-subunits in K^+ channel activity remains controversial (for review see Dunlap et al., 1987; Brown and Birnbaumer, 1990).

In addition to K^+ channels, Yatani and colleagues (1987 & 1988*c*) also demonstrated that activation of G-proteins stimulates dihydropyridine-sensitive mammalian cardiac Ca^{2+}-channels (for a detailed review see Trautwein and Hescheler, 1990). However,

in the central nervous system, activation of receptors coupled to G-proteins has been shown to inhibit the voltage-dependent Ca^{2+} channels. In embryonic chick sensory neurons, Dunlap and Fischbach (1981) demonstrated that many neurotransmitters such as noradrenaline, adenosine, opiates, and GABA inhibit Ca^{2+} spikes after activating G proteins. These agonists-induced activation of G protein-mediated suppression of Ca^{2+} currents have recently been shown to exist in a variety of cells including: a) sensory neurons of chick (Deisz and Lux, 1985; Forscher et al., 1986; Holz et al., 1986; Marchetti et al., 1986) and rat (Dolphin et al., 1986; Dolphin and Scott, 1987; Green and Cottrell, 1988; Schroeder et al., 1989), and b) sympathetic ganglion cells of chick (Marchetti et al., 1986).

In rat sympathetic neurons, activation of muscarinic receptors selectively reduced Ca^{2+} currents (Wanke et al, 1987). This observation was later confirmed by Toselli and colleagues using cultured hippocampal neurons (Toselli and Lux, 1989; Toselli et al., 1989). Moreover, this effect can be mimicked by intracellular application of the non-hydrolysable GTP analog GTPγS, resulting in permanent reduction of Ca^{2+} currents.

Non-hydrolysable GTP-analogues such as guanosine 5'-0-(3-thiotriphosphate) (GTPγS) and guanyl-5'-yl imidodiphosphate [Gpp(NH)p] have been shown to permanently activate G-proteins. Alternatively, permanent activation of G-proteins can be achieved by cholera toxin which ribosylates the α-subunits (α_s) of G_s at the arginine residue near the GTPase (Sullivan et al., 1987). Conversely, another bacterial toxin, pertussis toxin, ribosylates the cystein residue of the α-subunits (α_i and α_o) of G_i, thereby uncoupling

G-proteins from their receptors (Hsia et al., 1984; Vallar and Meldolesi, 1989; Sullivan et al., 1987). Because of their selective actions on the G-proteins, these two toxins have become an invaluable tool for the characterization of different G-protein subtypes. Nevertheless, not all G-proteins (e.g., G_p) are sensitive to cholera toxin and pertussis toxin.

Owing to the extensive information available on various G-proteins in signal transduction (see Gilman, 1987; Freissmuth et al., 1989; Ross, 1989; Birnbaumer et al., 1990; Simon et al., 1991), a detailed review of each subtype will not be included in this Introduction. Nevertheless, I shall focus on the functional role of G_p from a neuronal perspective, because the mystery surrounding the role of this G-protein in glutamatergic transmission is just beginning to unravel.

1.7.2 Activation of phospholipase C by G-proteins

The first clue to a possible interaction between G-proteins and receptors coupled to phosphoinositide metabolism was obtained from studies showing that addition of guanine nucleotides reduces receptor affinity for agonists such as vasopressin (Cantau et al., 1980), and epinephrine (Yamada et al., 1980; Goodhardt et al., 1982). Subsequent studies on permeabilized cells or isolated membranes have established the concept of PLC activation by receptors coupled to G-proteins. This hypothesis was supported by the observations that addition of GTP or non-hydrolysable GTP analogues stimulates phosphoinositide metabolism, and that the end products of this reaction trigger the Ca^{2+}-dependent secretory

process (Gomperts, 1983; Haslam and Davidson, 1984*a* & *b*; Oetting et al., 1986). Furthermore, the stimulatory effects of these non-hydrolysableGTP analogues was blocked by GDPβS, which permanently inactivates G-proteins.

In recent years, Sladeczek et al. (1985) demonstrated that activation of a novel glutamate receptor stimulates the formation of inositol phosphates in cultured striatal neurons. This observation was later confirmed by Sugiyama and company (1987 & 1989) who injected the Xenopus oocytes with rat brain mRNA expressing this novel glutamate receptor, which activates G-protein-mediated phosphoinositide metabolism. This subtype of glutamate receptors is now known as the metabotropic glutamate receptor (mGluR).

Activation of mGluRs by glutamate has been reported from studies using brain slices (Nicoletti et al., 1986*a*; Schoepp and Johnson, 1988; Palmer et al., 1988), neuronal cultures (Nicoletti et al., 1986*b*), gial cultures (Pearce et al., 1986), cultured hippocampal neurons (Murphy and Miller, 1988, 1989; Furuya et al., 1989), and reconstituted brain membranes (Recasens et al., 1987).

It is thus well established that activation of mGluR by glutamate stimulates phospholipase C and subsequently causes the breakdown of membrane phosphoinositides. Consequently, the major end products of phospholipase C activation are: a) diacylglycerol (DAG), which activates protein kinase C (PKC); and b) inositol-1,4,5-triphosphate (IP_3), which triggers Ca^{2+} release from cytosolic storage pools.

1.7.2.i Inositol trisphosphate

Intracellular free Ca^{2+} can also be increased - in addition to Ca^{2+} influx via the high voltage-activated Ca^{2+} channels - by activating membrane phosphoinositide turnover, as initially postulated by Michell (1975). This hypothesis was later confirmed by various groups showing that in many cell types, inositol trisphosphate (IP_3), resulting from the hydrolysis of phosphatidylinositol-4,5-bisphosphate(PIP_2) catalysed by phospholipase C (PLC), can stimulate Ca^{2+} release from non-mitochondrial Ca^{2+} storage pools or a 'high-affinity microsomal' store (Streb et al., 1983; Berridge and Irvine, 1984; Fisher and Agranoff, 1987; Berridge, 1987). Spät et al. (1986) believed that this process is initiated when the newly synthesized IP_3 binds to a specific binding site at the cytosolic surface of the 'high-affinity microsomal' membrane, thereby triggering the opening of microsomal Ca^{2+} channels.

IP_3 can be further phosphorylated by intrinsic inositol kinases to produce IP_4, IP_5, and IP_6 (Bansal and Majerus, 1990; Rana and Hokin, 1990). Of the three subsequent inositol phosphates produced, IP_4 has been shown to directly stimulate Ca^{2+} influx, thereby potentiating the response to IP_3 (Hansen et al., 1986; Irvine and Moor, 1986 & 1987; Putney, 1986).

1.7.2.ii Diacylglycerol

The other major end-product of phosphoinositide metabolism is the formation of diacylglycerol (DAG). This second messenger exerts its regulatory role on protein kinase

C from within the plasma membrane. The life span of DAG is relatively brief owing to the fact that it is rapidly metabolised by lipases to monoacylglycerol and then to free arachidonate and glycerol (Rana and Hokin, 1990). Arachidonic acid, as generated from this reaction or the metabolic action of phospholipase A_2, can subsequently have a variety of actions, such as activation of K channels (Kim and Clapham, 1989; Kim et al., 1989; Kurachi et al., 1989*a* & *b*; Ordway et al., 1989) or stimulation of transmitter release after diffusion to the presynaptic nerve terminals (Piomelli et al., 1987*a* & *b*; Lynch and Voss, 1990; O'Dell et al., 1991). Moreover, arachidonic is itself the precursor of some important potentially neuroactive agents such as prostagladin, and leukotrienes; these oxidized arachidonate derivatives are collectively termed eicosanoids (Corey et al., 1980; Shimizu and Wolfe, 1990). Alternatively, DAG can be phosphorylated by diacylglycerol kinase to form phosphatidic acid, which is then recycled for the synthesis of phosphoinositides.

Diacylglycerols with the 1,2-*sn* configuration and containing unsaturated fatty acids are the most potent activators of PKC; and the other active forms of DAG also having the 1,2-*sn* configuration but with various fatty acids of different chain lengths (Mori et al., 1982). The two stereoisomers (2,3-*sn*-diacylglycerol and 1,3-diacylglycerol), however, have no effect on PKC, thus suggesting a high degree of ligand-receptor specificity for PKC activation (Rando and Young, 1984; Boni and Rand, 1985).

Additional PKC activators which share a similar structural configuration with DAGs and are able to permeate cell membranes include: a) synthetic compounds such as

1-oleoyl-2-acetylglycerol,1,2-dioctanoylglycerol,1,2-didecanoylglycerol(Kaibuchi et al., 1982 & 1983; Lapetina et al., 1985; Davis et al., 1985); and b) tumour promoting phorbol esters such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), and phorbol 12, 13-diacetate (PDAc; Castagna et al., 1982; Yamanishi et al., 1983; Niedel et al., 1983). All these DAG analogues activate PKC in the absence of intracellular Ca^{2+} accumulation (Kaibuchi et al, 1981).

1.7.3 Protein kinase C

Protein kinase C (PKC) was initially purified from bovine cerebellum in the late seventies (Takai et al., 1977; Inoue et al., 1977), and subsequently found in all tissues and organs (Kuo et al., 1980). Activation of PKC requires both high $[Ca^{2+}]_i$ (approximately 100-fold greater than at rest) and phosphatidyl serine to form a ternary complex (Takai et al., 1979). However, diacylglycerol can substantially reduce the Ca^{2+} requirement to within a physiological range ($\sim 0.1\ \mu M$); and together with phosphatidyl serine, they form a quaternary complex (Takai et al., 1979; Kirk et al., 1981). Therefore under resting condition, and in the absence of Ca^{2+} influx through either HVA Ca^{2+}- or mGluR-gated channels, there is a basal PKC activity due to an ongoing production of DAG, consequent to the metabolism of phosphoinositides or other phospholipids.

1.7.3.i Members of PKC family

Recent developments in molecular cloning techniques have led to the identification

of seven subspecies of PKC from several mammalian tissues by screening a variety of complementary DNA (cDNA) libraries. The first four subspecies isolated from cDNA libraries are α, β_I, β_{II}, and γ (Parker et al., 1986; Coussens et al., 1986; Knopf et al., 1986). Subsequent screening from a rat brain library, using a mixture of α, β_{II}, and γ cDNA as probes, has yielded three other subspecies: δ, ε, and ζ (Ono et al., 1987, 1988).

All seven PKC subspecies are composed of a single polypeptide chain, which is then subdivided into the regulatory domain (near the amino-terminal) and the protein kinase (or catalytic) domain (near the carboxy-terminal). The polypeptide chain of PKC subspecies α, β_I, β_{II}, and γ contains four conserved regions (C_1-C_4) and five variable regions (V_1-V_5); whereas the other three PKC subspecies δ, ε, and ζ lack conserved region C_2. Moreover, C_1 and C_2 regions are situated in the regulatory domain, and have been postulated to bind DAG, Ca^{2+}, and phospholipid. The conserved region C_3 has an ATP-binding sequence; and the function of C_4 region is still unknown.

1.7.3.ii Expression and localization of different PKC subspecies

Using various radioactive labelling and *in situ* hybridization techniques, uneven distributions and expressions of all PKC subspecies were observed in both neuronal and non-neuronal tissues (Nagle and Blumberg, 1983; Worley et al., 1986; Coussens et al., 1986; Brandt et al., 1987). The only exception is γ-PKC which, so far, has been found predominantly in all regions of the brain including the hippocampus, cerebral cortex, and amygdaloid complex, as well as the spinal cord (Saito et al., 1988; Nishizuka, 1988) .

Moreover, it is concentrated in GABA-containing nerve terminals; which thus led to the suspicion that activation of γ-PKC may modulate GABA release (Shuntoh et al., 1989; Taniyama et al., 1990). Arachidonic acid is more potent than DAG in activating γ-PKC, which subsequently induces transmitter release (Taniyama et al., 1990).

Enhanced transmitter release by brain PKC has been demonstrated in the hippocampus (Allgaier and Hertting, 1986; Feuerstein et al., 1987; Malenka et al., 1987; Versteeg and Florijn, 1987; Agopyan et al., 1992), caudate nucleus (Tanaka et al., 1986; Bartmann et al., 1989; Chandler and Lesli, 1989; Weiss et al., 1989), and cerebral cortex (Peterfreund and Vale, 1984; Shuntoh et al., 1988; Friedman and Wang, 1989).

1.7.3.iii Modulation of transmitter release by PKC

One of the mechanisms underlying PKC-induced facilitation of transmitter release is through phosphorylation of B-50, a membrane bound protein (Niedel and Blackshear, 1986; Dekker et al., 1989 & 1990). B-50 is located exclusively on the intracellular side of neuronal plasma membrane (McGuire et al., 1988; Gorgels et al., 1989); and it has a high binding affinity for calmodulin, under two conditions: a) in the absence of Ca^{2+} (Andreasen et al., 1983; Cimler et al., 1987), and b) in its dephosphorylated form (Alexander et al., 1987).

These stringent calmodulin binding requirements of B-50 are consistent with the role of both B-50 and calmodulin (more below) in regulating transmitter release. That is, during nerve terminal depolarization, accumulation of $[Ca^{2+}]_i$ would reduce the B-50

binding affinity for calmodulin, thereby liberating membrane bound calmodulin. The elevation in cytosol calmodulin concentration would trigger the calmodulin-dependent phosphorylation cascade, resulting in calmodulin-mediated transmitter release (more below). The free B-50 phosphoprotein is then phosphorylated by PKC, which appears to facilitate membrane fusion between the transmitter-containing vesicles and plasma membrane. However, the exact mechanism underlying this process, as induced by PKC-dependent B-50 phosphorylation, remains to be resolved.

Inactivation of the phosphorylated B-50 protein occurs through dephosphorylation catalysed by calcineurin phosphatase, which is activated by calmodulin (Liu and Storm, 1989; Schrama et al., 1989).

1.7.4 Calmodulin

In addition to the activation of calcineurin phosphatase, calmodulin also initiates a number of other phosphorylations that influences transmitter release. Moreover, like PKC, the activation of calmodulin is highly dependent on Ca^{2+}. Calmodulin is one of many Ca^{2+}-binding proteins in the cytoplasm; and its activation leads to phosphorylation of many Ca^{2+}/calmodulin dependent-, as well as cAMP-dependent kinases (see Fujisawa et al., 1984, Kennedy, 1987, and Trimble et al., 1991 for detailed reviews).

Of the five Ca^{2+}/calmodulin-dependent kinases isolated to date, Ca^{2+}/calmodulin-dependent kinase II (CaM kinase II) differs from all others by virtue of its abundance in neuronal tissues such as cerebral cortex, brainstem, and cerebellum

(Yamauchi and Fujisawa, 1981), as well as its broad substrate specificity (Yamauchi and Fujisawa, 1980). However, I shall only focus on two substrates which play a significant role in modulating transmitter release. These substrates are the synapsins and microtubule-associated protein-2 (MAP-2).

1.7.4.i Synapsins

Synapsins form a group of phosphoproteins found exclusively in nerve terminals. Thus far, a total of four homologous synapsins have been characterized. Synapsins I*a* and its isoform I*b* are collectively called synapsin I; and synapsins II*a* and its isoform II*b* are collectively called synapsin II. The *a* and *b* isoforms of either synapsin I or II are the end products of alternative splicing; and synapsin I and II are encoded by two distinct genes (Südhof et al., 1989). Owing to their association with the synaptic vesicles (Forn and Greengard, 1978; Browning et al., 1987; Benfenati et al., 1989), when in the phosphorylated form, these synapsins have been postulated to facilitate transmitter release.

This hypothesis was first tested by Llinás et al. (1985) who injected various forms of phosphorylated synapsins into the gaint squid axon near synapse. It was shown in that injections of the de-phosphorylated synapsin I substantially reduced both the evoked EPSPs and the rate of occurence of spontaneous miniature EPSPs. Subsequent phosphorylation of synapsin I by CaM kinase II reversed the effects of the de-phosphorylated form. More recently, these observations were substantiated by studies using rat brain synaptosomes loaded with autophosphorylating CaM kinase (Nichols et al., 1990).

Indeed, one attractive feature of CaM kinase is its ability to initiate autophosphorylation in a Ca^{2+}/calmodulin-dependent manner; once the autophosphorylation process starts, Ca^{2+}/calmodulin is no longer needed to maintain the phosphorylated state (Fukunaga et al., 1982; Bennett et al., 1983; Goldenring et al., 1983; Kuret and Schulman, 1985). Therefore activation of CaM kinase II greatly prolongs the Ca^{2+}-dependent transmitter release initiated by a transient increase in $[Ca^{2+}]_i$.

1.7.4.ii Microtubule-associated protein-2

The other substrate which is also phosphorylated by CaM kinase is the microtubule-associated protein-2 (MAP-2; Yamauchi and Fujisawa, 1982). This phosphoprotein is found most abundantly in brain tissue (Olmsted and Borisy, 1973). In particular, MAP-2 is located in the cell body (Izant and McIntosh, 1980; Matus et al., 1981) and in dendrites (Matus et al., 1981; Caceres et al., 1983). Once phosphorylated by Ca^{2+}/calmodulin CaM kinase, these proteins inhibit the assembly of microtubules and promote disassembly of polymerized microtubules, resulting in transmitter release (Jameson et al., 1980; Burke and DeLorenzo, 1981 & 1982; Jameson and Caplow, 1981).

1.8 Summary of Ca^{2+}-Dependent Transmitter Release

In the latter half of this Introduction, I have reviewed most of the mechanisms associated with neurotransmitter release. Briefly, when an action potential invades the

nerve terminal, it activates HVA Ca^{2+} channels, resulting in a transient elevation of $[Ca^{2+}]_i$. Accumulation of $[Ca^{2+}]_i$ can either directly stimulate transmitter release or activate, as an ubiquitous second messenger, membrane bound enzymes such as phospholipase C, or multi-functional protein kinases such as protein kinase C and calmodulin. Each of these protein kinases is ultimately responsible for a secondary, or perhaps more sustained, accumulation of $[Ca^{2+}]_i$ that persists long after the initial depolarization. Hence, activation of these second messengers has long been associated with the phenomenon of long term potentiation (LTP), which is an experimental paradigm for memory processes.

1.9 Rationale

Brain gangliosides, in particular monosialoganglioside (GM1), have been implicated in the modulation of synaptic transmission, by virtue of their eminent presence in nerve terminals. Several investigators have demonstrated that an increasing membrane concentration of GM1, produced either by enzymatic treatment with neuraminase or incorporation of exogenous GM1, decreases the threshold for the induction of LTP (Römer and Rahmann, 1979; Wieraszko and Seifert, 1985; Ramirez et al., 1990). Moreover, GM1 has been shown to interact with second messengers such as CaM kinase (Cimino, 1987; Fukunaga et al., 1990; Higashi et al., 1992; Higashi and Yamagata, 1992), and protein kinase C (Kreutter et al., 1987; Agopyan et al., 1992), which all have been

implicated in the induction of LTP.

Although numerous hypotheses have been postulated by various investigators, the mechanisms underlying the improvement in efficacy of synaptic transmission induced by GM1 are still unclear. In fact, no one has yet attempted to address these issues from an electrophysiological perspective. Hence the goal of this thesis project was to elucidate the mechanism(s) of GM1 action on synaptic transmission, using rat hippocampal slices.

1.10 References

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CHAPTER 2

2.1 METHODS

In vitro hippocampal slices have been widely used in the field of Neuroscience to investigate various aspects of brain physiology. Although hippocampal slices are still far from an ideal *in vivo* preparation, they offer distinct advantages over other preparations such as cultured or acutely dissociated cells. The most important advantages of using brain slices are a) precise control of extracellular milieu, and b) functionally intact circuitry, at least in two dimensions. The latter is essentially lacking if one chooses cultured or acutely dissociated cells. Another disadvantage of cultured neurons is that the expression and modulation of the same ligand-gated receptors or channels are sometimes quite different from that found in intact cells, which may lead to erroneous conclusions. As for the dissociated neurons, much of their dendritic tree is removed as a consequence of the mechanical dissociation process. Moreover, it is uncertain what effect the enzymatic treatment has on the functional integrity of the cells.

During the past few decades, a tremendous volume of information on the anatomical organization, neuronal properties, and synaptic transmission has been obtained from the hippocampal slice preparation. As a result of numerous electrophysiological studies, widely ranging from single channel recordings of microscopic currents (from individual voltage- or ligand-gated channels) to macroscopic currents produced by multiple excitatory and inhibitory synapses, as well as extracellular field recordings, there have been great advances in our understanding of the intricacy of the hippocampal pyramidal cell properties

and their synapses.

However, in spite of the advantages of brain slices, one should still bear in mind that there are disadvantages as well. For example, one cannot study interactions between other parts of the brain and the hippocampal slices since during the slicing process, connections are destroyed. Moreover, there is an oxygen gradient difference created by slice thickness; i.e., cells near the surface may receive adequate oxygenation due to the constant fluid flow whereas the deeper layers may receive relatively little oxygen. However, the problem can be partially corrected by having different slice thickness. As for the hippocampal slices, the optimal thickness has been empirically determined to be around 400 to 450 μm thick.

In this thesis, we took advantage of the lamellar organization of fibres in transverse hippocampal slices to study the role of the ganglioside GM1 in various synaptic interactions impinging on the pyramidal neurons. The well-defined anatomical organization of the hippocampus allows easy visualization and identification of the perikarya for intracellular penetration with microelectrodes (Warburg, 1930; Yamamoto and McIlwain, 1966; Skrede and Westgaard, 1971).

2.2 Slice Preparation

Male Sprague Dawley rats, weighing between 120 to 150 g, were used for all the experiments described in this thesis. An animal was initially anaesthetized with halothane

in a closed box. Following the loss of righting reflex, the rat was decapitated with a scalpel. After a frontal craniotomy, the brain was immediately removed with a curved spatula and placed in pre-cooled (~4°) artificial cerebrospinal fluid (ACSF), oxygenated with 95/5% gaseous mixture of O_2/CO_2 respectively. The constituents of the ACSF were (in mM): NaCl 124, KCl 3, $MgCl_2$ 2, $CaCl_2$ 2, NaH_2PO_4 1.25, $NaHCO_3$ 26, and glucose 10.

Both hippocampi were dissected and transverse slices of approximately 450 μm thick were cut with a McIlwain tissue chopper. Two slices were selected and placed on a nylon mesh in an interface-recording chamber (Schwartzkroin, 1975; Haas et al., 1979). For some extracellular studies, slices were also placed in a submersion-recording chamber (White et al., 1978), where the slices were completely immersed in oxygenated ACSF. In either chamber, the slices were perfused continuously at a constant rate of 2-3 ml/min with oxygenated ACSF (pH 7.4). The temperature of ACSF was usually set at 33° instead of the normal body temperature (37°) since most investigators have found that the slices are maintained longer and healthier at lower temperatures. Moreover, at higher temperatures, there is a tendency towards the development of epileptiform discharges. The remaining slices were kept in the holding chamber and oxygenated with 95/5% O_2/CO_2 at room temperature. Slices were allowed to stabilize in the recording chamber for approximately one hour before electrical recordings began.

2.3 Recording Chambers

For all of the intracellular and some extracellular studies described in this thesis, we used a Haas-type recording chamber, purchased from Medical System Corp. of USA. As illustrated in figure 2.1, the base unit contains distilled water and an aerating tube to provide humidified air to the atmosphere immediately above the recording chamber. The perfusate can be warmed to the desired temperature as it passes through the heated water bath. A temperature probe, located in the recording chamber, regulates and maintains ACSF temperature via a feedback loop. The Haas recording chamber is designed to allow superfusion of the brain slice (Schwartzkroin, 1975; Haas et al., 1979), and provides reasonably rapid exchange of perfusion fluids (running in contact with the lower surface of the slice). With the perfusion rate usually set at 2 ml/min, near complete replacement of the perfusate in the recording chamber was achieved within 5 to 10 min. For many of the cells recorded, the chamber provided stable intracellular recordings for well over 4 hours.

2.4 Recording Techniques

2.4.1 Types of recording electrodes

Single-barrel glass capillaries (with filament) were purchased from World Precision Instrument (WPI), Inc. USA. For extracellular studies, the diameter of the capillaries was

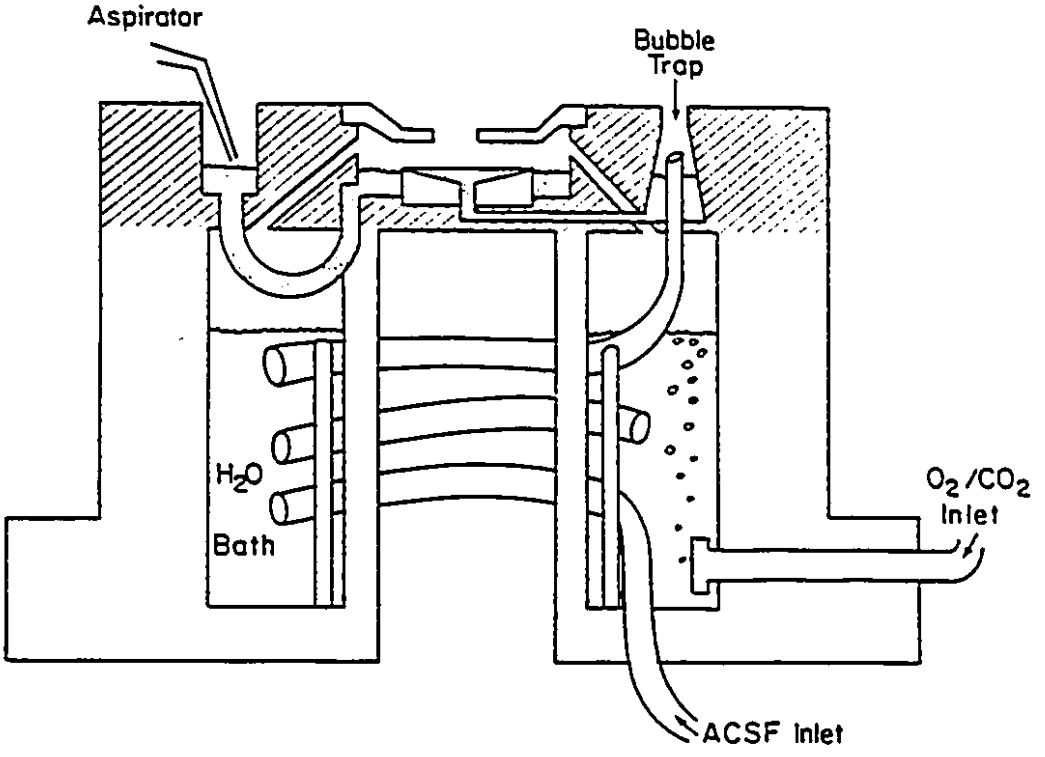

Figure 2.1: A schematic diagram of an interface chamber.

1.5 mm o.d., and they were subsequently pulled by a Narishige micropipette puller (Model PN-3 of Narishige Scientific Instrument, Japan). Smaller outer diameter capillaries (1.2 mm o.d.) were drawn out by a Flaming Brown micropipette puller (Model P-80/PC of Sutter Instrument Co., USA) for all the intracellular studies.

2.4.2 Iontophoretic electrodes

In some experiments, L-glutamate (500 mM; pH 7.0) was applied iontophoretically via a double-barrel glass micropipette, made by gluing two single-barrel glass capillaries (1.5 mm o.d. from WPI) together with epoxy glue. They were then drawn out by a vertical Narishige micropipette puller (Model PE-2 of Narishige Scientific Instrument, Japan).

2.4.3 Extracellular studies

Conventional extracellular and intracellular recording techniques were used in conjunction with an Axoclamp-2 amplifier (Axon Instruments Ltd.). Extracellular field potentials were recorded from the stratum pyramidale and stratum radiatum, using single-barrel glass micropipettes containing 2 M NaCl (electrode resistance - R_e - = 2-10 MΩ). The electrode was mounted on an electrode holder driven by a Nano-stepper (Model type B, W. Germany). The electrode was usually lowered to the depth of 120-150 μm. Orthodromically evoked field potentials were generated in both regions following stimulation of the Schaffer collaterals by an insulated NiCr wire. The

stimulation frequency was set at 0.1 Hz by a Grass S48 stimulator (Grass Medical Instrument, USA), and the intensity was adjusted to give 50% of the maximal evoked amplitudes.

2.4.4 Intracellular studies

2.4.4.i Current clamp

Neurons of the CA1 and CA3 pyramidal layer were impaled with single-barrel glass micropipettes containing one of the following solutions (all at 3 M): a) KCl (R_e = 40-60 MΩ), b) potassium acetate (R_e = 60-80 MΩ, pH 7.4), c) potassium methylsulfate (R_e = 80-100 MΩ), d) CsCl (R_e = 100-120 MΩ), or e) Cs acetate (R_e = 80-100 MΩ). Only neurons with stable resting potentials (V_m > -65 mV), input resistance (R_N) greater than 30 MΩ, and action potentials greater than 85 mV were selected for GM1 administration. In addition, a pronounced sag, characteristic of pyramidal CA1 and CA3 neurons, was observed in all the cells tested.

2.4.4.4.ii Voltage clamp

Using the single-electrode voltage-clamp (SEVC) technique (Brennecke and Lindemann, 1974*a* & *b*; Merickel, 1980; Finkel and Redman, 1984), deliberate attempts were made to produce recording electrodes with the lowest possible tip resistance (R_e) and a short electrode shank. These electrode attributes are crucial in determining a rapid decay rate of the voltage drop across the resistance of the electrode

caused by current injection into the cell through the micropipette, prior to the next sampled voltage. Although the maximum sampling frequency can be improved by the capacitance compensation circuitry of the Axoclamp-2 amplifier, a low R_e and electrode capacitance to ground will maximize the effective sampling frequency.

In our SEVC experiments, the sampling frequency was usually set at 4-5 kHz, the clamp gain at 3-8 nA/mV, and the output bandwidth at 0.3-1 kHz. The input waveform was continuously monitored to ensure that capacity neutralization was near optimal.

2.5 References

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CHAPTER 3

3.1 SYNAPTIC MODIFICATIONS BY MONOSIALOGANGLIOSIDE IN RAT HIPPOCAMPAL SLICES

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To be submitted

3.2 Summary

1. The effects of exogenous monosialoganglioside (GM1; 0.1 - 10 μM) on pyramidal CA1 and CA3 neurons of rat hippocampal slices were studied by extra- and intracellular recording techniques, using either current or single electrode voltage clamp. Extracellular field potentials were recorded from both stratum pyramidale and stratum radiatum of CA1 region. Intracellular recordings were obtained from CA1 as well as CA3 pyramidal neurons.

2. GM1 enhanced Schaffer collateral-evoked field EPSPs by increasing synaptic efficacy, as shown by a steeper EPSP versus afferent volley slope. In stratum pyramidale, GM1 consistently increased the population spike amplitude but did not significantly alter the relation between EPSPs and spikes (E/S coupling).

3. Intracellular recordings from CA1 pyramidal cellsshowed that GM1 selectively potentiates the excitatory synaptic inputs, while suppressing the inhibitory synaptic inputs.

4. The GM1-induced increase in excitatory synaptic activity is indicated by a) an increase in the frequency of spontaneous, kynurenate- and TTX-sensitive inward currents (sEPSCs), b) a rightward shift in the amplitude distribution of sEPSCs, and c) an increase in the amplitude of evoked excitatory postsynaptic potentials (EPSPs) and currents (EPSCs). The frequency and amplitude of the sEPSPs by GM1 were also increased in cells continuously superfused with 10 μM bicuculline.

5. In the presence of TTX, inward currents evoked repeatedly by brief iontophoretic application of glutamate (in stratum radiatum of CA1 region) were enhanced by GM1; the glutamate-responses reached a peak after 5 min, and returned to their initial level by 10 min, before the end of GM1 application.

6. In CA3 neurons, where spontaneous miniature EPSCs were readily recorded in the presence of TTX, GM1 increased their frequency and amplitude. These observations suggest a presynaptic, action potential-independent modulation of transmitter release by GM1.

7. In the absence of TTX, suppression of the inhibitory input to CA1 neurons was indicated by a reduction in the frequency of bicuculline-sensitive spontaneous outward currents (sIPSCs), and evoked inhibitory synaptic potentials (IPSPs) and currents (IPSCs). The slope of IPSC amplitude versus membrane potential plots was significantly reduced by GM1 administration, without significant change in the reversal potential.

8. But monosynaptic IPSPs - evoked in CA1 neurons by stimulating stratum radiatum in the presence of kynurenic acid - were not affected by GM1, since there was no significant change in the slope of the monosynaptic IPSP amplitude versus membrane potential plots. The lack of any direct effect of GM1 on the inhibitory synapses is further supported by the absence of changes in both the frequency and amplitude of TTX-insensitive spontaneous miniature IPSCs.

9. We therefore conclude that exogenous GM1 facilitates excitatory synaptic

transmission to pyramidal cells by increasing the release, and perhaps the binding, of the excitatory neurotransmitter(s); at the same time, GM1 blocks the polysynaptic inhibitory input, resulting in disinhibition, which ultimately may lead to the development of epileptiform activity.

3.3 Introduction

Gangliosides are sialo-glycolipids containing a hydrophobic ceramide moiety and a hydrophilic oligosaccharide moiety. These glycosphingolipids are present in a variety of cells (Cuatrecasas, 1973; Fishman et al., 1976; Moss et al., 1976; Fuxe et al., 1989), as well as neuronal membrane preparations (Toffano et al., 1980). Thus far, forty different species of gangliosides have been identified throughout the body. However, four major brain gangliosides are the monosialoganglioside (GM1), disialogangliosides 1a, 1b (GD1a, and GD1b respectively), and trisialoganglioside 1b (GT1b). These gangliosides are inserted in the plasma membrane in such manner that the hydrophobic portion interacts with the lipid bilayer, while the hydrophilic portion extends into the extracellular milieu (Morgan et al., 1973; Landa et al., 1981). Immunofluorescence has demonstrated that GM1 exists in high concentrations in nerve terminals (Fuxe et al., 1989). Hence, in view of GM1's location, numerous investigators have postulated that GM1 may have a powerful influence on synaptic transmission and plasticity, by modulating various enzymes involved in the second messenger systems (Rahmann et al., 1976; Wieraszko and Seifert, 1984).

Indeed, previous findings have demonstrated that incubation with exogenous GM1 or neuraminidase, which transforms polysialogangliosides into the monosialoganglioside GM1, improves the efficacy of synaptic transmission in frog spinal cord and fish optic tectum (Römer and Rahmann, 1979). In the CA1 subfield (Wieraszko and Seifert, 1985) and the dentate gyrus of rat hippocampal slices (Ramirez et al., 1990), GM1 enhances synaptic transmission following tetanic stimulation. In whole animal studies, chronic administration of exogenous GM1 during development improves performance in memory tasks (Fagioli et al., 1990), whereas administration of antiserum against brain gangliosides inhibits learning and memory processes (Karpiak et al., 1978*a*; Kobiler et al., 1976).

The mechanisms by which GM1 improves the efficacy of synaptic transmission are still unclear. Several have been postulated, including: a) alteration of plasma membrane ionic conductances, thus altering transmitter release (Burton and Howard, 1967), b) interactions with calcium (McDaniel and McLaughlin, 1985; Wolf and Irwin, 1991), protein kinase C and calcium calmodulin-dependent kinases, which have important roles as second messengers involved in neurotransmitter release, learning and memory (Cimino, 1987; Kreutter et al., 1987), and c) modulation of ligand-receptor binding (Hollmann and Seifert, 1986).

Hence this study was performed to elucidate GM1's action on synaptic transmission in the CA1 and CA3 subfields of the rat hippocampal slices, using both extra- and intracellular recording techniques.

Preliminary reports have been presented in abstract forms (Miu and Krnjević, 1990;

Miu and Krnjević, 1992).

3.4 Methods

3.4.1 Preparation of slices

Hippocampal slices were prepared and maintained by conventional techniques (Dingledine, 1984). Briefly, the brain of male Sprague-Dawley rats (150-200 g) was removed under halothane anaesthesia and immersed in pre-cooled (~4°) artificial cerebrospinal fluid (ACSF) that was oxygenated with 95/5% gaseous mixture of O_2/CO_2 respectively. The constituents of the ACSF were (in mM): NaCl 124, KCl 3, $MgCl_2$ 2, $CaCl_2$ 2, NaH_2PO_4 1.25, $NaHCO_3$ 26, and glucose 10.

After dissecting out the hippocampus, transverse slices, 450 μm thick, were cut with a McIlwain tissue chopper. Two to four slices were placed in an interface-recording chamber. Some extracellular recordings were also performed in a submersion-type chamber. The slices were perfused continuously at a constant rate of 2-3 ml/min with oxygenated ACSF at 33° (pH 7.4). The remaining slices were kept in the holding chamber and oxygenated with 95/5% O_2/CO_2 at room temperature. Slices were allowed to stabilize in the recording chamber for approximately one hour before the start of the experiment.

3.4.2 Recording techniques and arrangement

Conventional recording techniques were used in conjunction with an Axoclamp-2

amplifier (Axon Instruments Ltd.). Extracellular field potentials were recorded from the stratum pyramidale and stratum radiatum, using single-barrel glass micropipettes containing 2 M NaCl (electrode resistance - R_e - = 2-10 MΩ).

Neurons of the CA1 pyramidal layer were impaled with single-barrel glass micropipettes containing one of the following solutions (all at 3 M): a) KCl (R_e = 40-60 MΩ), b) potassium acetate (R_e = 60-80 MΩ, pH 7.4), c) potassium methylsulfate (R_e = 80-100 MΩ), or d) CsCl (R_e = 100-120 MΩ). A total of 65 CA1 and 5 CA3 pyramidal neurons with stable resting potentials, input resistance (R_N) greater than 30 MΩ and action potentials greater than 85 mV were selected for GM1 administration.

Fifty-three of the sixty-five CA1 pyramidal cells were impaled with micropipettes containing either 3 M potassium acetate or 3 M potassium methylsulfate to study spontaneous and evoked EPSCs and IPSCs respectively by single-electrode voltage clamp (SEVC). Five CA3 cells were voltage-clamped using glass micropipettes containing 3 M KCl to study sEPSCs. The sampling frequency was usually set at 4-5 kHz, the clamp gain at 3-8 nA/mV, and the output bandwidth at 0.3-1 kHz. The input waveform was continuously monitored to ensure that capacity neutralization was near optimal.

Orthodromically-evokedsynaptic responses were elicited by an insulated NiCr wire electrode placed in the stratum radiatum. Stimulation frequency was set at 0.1 Hz, and the intensity was adjusted to evoke half-maximal postsynaptic potentials. Spontaneous PSPs were recorded in the absence of orthodromic stimulation. In some experiments, excitatory inputs to CA1 neurons were suppressed by either cutting the connections between CA3 and

CA1 or by applying 1 μM TTX. Spontaneous miniature EPSCs (from CA3 neurons) and IPSCs (from CA1 neurons) were recorded in the presence of TTX (1 μM).

In twelve experiments, L-glutamate was applied iontophoretically in the stratum radiatum from a double-barrel electrode (containing Na^+-L-glutamate, 500 mM, pH 7.0) while recording from twelve CA1 neurons in the presence of 1 μM TTX. The current intensity and duration of glutamate ejection (by the push-pull method) were adjusted to elicit half-maximal responses. Control NaCl current, equal in intensity but of opposite polarity, was injected intermittently as a control for current artifacts. Most often, the duration and frequency of L-glutamate applications were 10 s and 0.01 Hz respectively.

Extracellular fields, and intracellular current- and voltage-clamp data were monitored continuously on a Gould pen recorder and on an oscilloscope. Traces were stored on an IBM AT computer for subsequent off-line analysis. The spontaneous PSPs were further amplified by an ORTEC (Model 4660) bandpass amplifier. The lower frequency was set at D.C., and the upper frequency at 20 KHz. The amplified signal was then recorded continuously by a Gould pen recorder in AC mode (low cutoff frequency 10 Hz and high cutoff frequency 100 Hz) in order to eliminate severe DC shifts at high gain.

All values are reported as means $\pm$ standard errors. Statistical analyses were performed in all the experiments by paired Student's t test unless indicated otherwise. The conventional significance level of $p < 0.05$ was adopted for all statistical comparisons.

3.4.3 Solutions

Concentrated stock solutions of tetrodotoxin (TTX), tetraethylammonium chloride (TEA), (-)-bicuculline methiodide (BMI), and CsCl (all from Sigma Inc.) were made in distilled water, and diluted to their final concentration in the perfusing medium. Kynurenic acid (Sigma Inc.) was added directly to the ACSF.

Monosialoganglioside GM1 (M.Wt. 1569) was dissolved in distilled water and further diluted to a final concentration of 0.1, 1, or 10 μM in the perfusing medium. In most cases, we used only 1 μM GM1, because varying the concentration did not produce clearly dose-dependent responses. Higher doses were, nevertheless, undesirable owing to the possible formation of micelles in aqueous solution (Toffano et al., 1980). GM1 was a gift from Dr. Toffano at FIDIA Institute (Abano Terme, Italy).

3.5 Results

3.5.1 Extracellular studies

3.5.1.i Effects of exogenous GM1 on extracellular field potentials

Field potentials in stratum pyramidale were evoked by orthodromic stimulation of the Schaffer collaterals, submaximal for population spikes. GM1 (1.0 μM) increased the initial slope of the positive wave (representing the dendritic EPSP) by 11.2 $\pm$ 4.2% ($n = 58, p = 0.01$). Figure 3.1A shows an example of GM1-induced augmentation of the positive wave slope with a concomitant increase in the amplitude of the population spike

(by 13.3 ± 3.4%; n = 59, p < 0.001). In all slices tested, prolonged incubation or superfusion with GM1 resulted in the generation of multiple spikes, in response to submaximal orthodromic stimulation (data not shown).

In eight slices, the effects of GM1 on the relation between EPSPs and population spikes (E/S coupling) were examined over a wide range of stimulus intensities by plotting the population spike amplitude as a function of the positive wave slope (dV/dt). Figure 3.1B is an example of such a plot which shows that GM1 did not significantly alter the E/S coupling. In six out of eight slices, however, (as in Fig. 3.1B) there was some increase in maximum population spike amplitude.

In agreement with their effects in the stratum pyramidale, bath applications of GM1 (1.0 μM) increased the dendritic EPSP (Fig. 3.2, insert), recorded from the stratum radiatum, by 33.4 ± 5.7% (control mean value = 2.0 ± 0.4 mV, n = 7, p < 0.005). Also shown in this figure is that the slope of dendritic EPSP amplitude versus afferent volley became steeper, comparable increases in synaptic efficacy were seen in all slices tested.

3.5.2 Intracellular studies

3.5.2.i Effects of GM1 on passive membrane properties

The mean resting potential (V_m) was -74 ± 3.3 mV (n = 10) in standard ACSF. When the cells were superfused with 1 μM GM1, they were consistently depolarized (by 5.7 ± 0.7 mV; n = 7, $P < 0.0005$). This depolarization was associated with a slight but

statistically not significant increase in the input resistance (R_N) - measured at control V_m - by 7.2 ± 4.7% (n = 11). In some cells, there was a transient increase in cell firing during the depolarizing phase (data not shown).

3.5.2.ii Effects of GM1 on evoked postsynaptic potentials and currents

In eight CA1 neurons, postsynaptic potentials were recorded with potassium acetate- or methylsulfate-filled glass micropipettes in response to orthodromic stimulation of the Schaffer collaterals (subthreshold for post-synaptic spikes).

GM1 had opposite effects on excitatory and inhibitory responses. Thus GM1 (1.0 μM) reversibly augmented the amplitudes of evoked EPSPs, by 32.1 ± 8.0% (n = 7, from a mean control value of 6.3 ± 1.1 mV, $P < 0.005$; Fig. 3.3A). But - as also evident in Fig. 3.3A - both the early and late phases of evoked IPSPs were reduced by GM1: the early IPSP by 34.5 ± 3.3% (n = 10, $P < 0.0005$), and the late IPSP by 37.4 ± 9.3% (n = 4, $p < 0.025$).

Similar changes were observed during SEVC recording: 1.0 μM GM1 enhanced the evoked EPSCs by 41.0 ± 18.7% (n = 10, $P < 0.05$); whereas the early and late evoked IPSCs were reduced by 31.1 ± 7.2% (n = 15, $P < 0.001$) and 27.2 ± 5.5% (n = 10, $P < 0.0005$) respectively (Fig. 3.3B).

In order to assess whether or not GM1's action on the evoked IPSCs is voltage-dependent, the holding potential was varied over a wide range. As shown in figure 3.3C, 1 μM GM1 reduced the slope of the current-voltage plots, but the IPSC reversal

potential did not obviously change.

3.5.2.iii Effects of GM1 on isolated inhibitory inputs

In eight CA1 neurons, the GM1-mediated reduction of evoked IPSPs was further studied in the absence of any excitatory synaptic inputs. These cells were recorded with glass micropipettes containing 3 M K-methylsulfate, and the bipolar stimulating electrode was placed in the stratum radiatum within 0.5 mm of the recorded cell.

Initially, the orthodromic stimulation (subthreshold for a post-synaptic spike) elicited a typical triphasic waveform; i.e., an EPSP followed by early and late IPSPs. The EPSP was then blocked by 2 mM kynurenic acid to reveal a pure monosynaptic IPSP. Owing to pronounced anomalous rectification, the voltage-dependence of the IPSPs could not be studied over a wide range of potentials and we could not get a clear reversal (Fig. 3.4C, open circles). To overcome this problem, Cs^+ (4 mM) was added to the perfusate containing kynurenic acid (Fig. 3.4). Under these conditions, GM1 (1 μM) did not have any effect on the early or late components of monosynaptic IPSPs, measured respectively at 50 ms and 160 ms from the stimulus artifact (Fig. 3.4B). In addition, the voltage-current plots (Fig. 3.4C) showed no change in either the slope or the reversal potential during GM1 perfusion. The lack of GM1 effect on the monosynaptic IPSPs was observed in all cells tested.

3.5.2.iv Effects of GM1 on glutamate-evoked inward currents

We also looked for a possible interaction between GM1 and postsynaptic excitatory receptors by applying L-glutamate iontophoretically to 12 CA1 neurons, in the presence of TTX. Figure 3.5A shows typical responses to brief pulses of glutamate applied in the stratum radiatum at regular intervals. Also visible in this figure are spontaneous miniature inward transients, most likely GABA-mediated spontaneous miniature IPSCs (Alger and Nicoll, 1980; more below).

As shown in figure 3.5A2, the glutamate-evoked response was potentiated during the first few minutes of GM1 perfusion. However, during prolonged perfusion of GM1 the glutamate current diminished as shown in figure 3.5A3; and this cell showed a further fading of the glutamate current even when GM1 was washed out (Fig. 3.5A4).

Figure 3.5B is a summary of the time course of GM1 action on glutamate currents recorded in twelve CA1 neurons. The holding potentials (V_H) were -60 to -70 mV. For nearly 10 min, GM1 significantly increased the glutamate current: by 17.0 $\pm$ 7% at 4 min ($n = 12$, $P < 0.01$), and 17.0 $\pm$ 5.4% at 6 min ($n = 12$, $P < 0.01$). After 10 min, the glutamate-current returned to control level, as illustrated in figure 3.5A. Statistical signifiance was determined by one way ANOVA ($F_{obt} = 3.05$) for various times during GM1 perfusion, and individual times were compared to control (100 $\pm$ 1.6%, $n = 76$) by t-test for multiple comparisons.

3.5.3 Spontaneous postsynaptic currents (recorded in the absence of TTX)

3.5.3.i Spontaneous EPSCs and IPSCs recorded in ACSF

In the absence of orthodromic stimulation of the Schaffer collaterals, spontaneous EPSCs and IPSCs (sEPSCs and sIPSCs respectively) were recorded by SEVC, from five CA1 neurons, with either potassium acetate- or methylsulfate-filled micropipettes.

As described in a previous report (Agopyan et al, 1992), at V_H -50 to -60 mV, the sIPSCs manifested themselves as outward transients, and the sEPSCs as inward transients. The outward transients were sensitive to bicuculline (Fig. 5 of Agopyan et al, 1992), and the inward transients were sensitive to kynurenic acid (cf. CA3 cells of Fig. 3.8); this confirmed their identity as IPSCs and EPSCs respectively.

The outward transients (sIPSCs) were probably mediated by Cl^- since the frequency and amplitude of the outward spikes were much reduced or reversed at V_H -75 mV (data not shown). In ten CA1 cells that were recorded with 3 M CsCl micropipettes (i.e. chloride loaded), only inward transients were observed and there was no reversal over the ranges of V_H -50 to -75 mV (data not shown).

Cutting the connections between CA3 and CA1 substantially reduced both the inward and outward transients (in 8 out of 10 neurons), thus suggesting a predominance of action potential-dependent transmitter release (Agopyan et al, 1992). In addition, superfusing the slices with TTX abolished kynurenic-sensitive sPSPs; however, as found by previous observers (Alger and Nicoll, 1980; Ropert et al, 1990), we could still detect bicuculline-sensitive miniature sIPSPs (see below and Fig. 3.9). The selective detection

of the miniature sIPSPs is presumably due to the proximity of inhibitory synapses to the soma (Andersen et al., 1964*a*); in contrast to the much more distally located excitatory synapses (Masukawa and Prince, 1984).

3.5.3.ii Effects of GM1 on spontaneous EPSCs and IPSCs

As reported previously (Agopyan et al, 1992), the frequency and amplitude of the outward transients (IPSCs) were consistently reduced by GM1, whereas the inward transients (EPSCs) were consistently enhanced by GM1.

Quantitative analysis of the frequency and amplitude of sIPSCs showed that 1 μM GM1 shifted the distribution of sIPSC amplitudes to the right, the peak moving from 15 pA in ACSF to 25 pA ($n = 11, \chi^2_{obt} = 18.4, P < 0.05$). In addition, the average frequency of sIPSCs was reduced from 0.36 ± 0.11 Hz ($n = 6$) in control to 0.16 ± 0.03 Hz ($n = 6$). These effects of GM1 were fully reversible after 20 min of washing.

Similarly, GM1 significantly shifted the distribution of the sEPSC amplitudes to the right, the peak moving from 10 pA in ACSF to approximately 35 pA ($n = 17, \chi^2_{obt} = 204.1, P < 0.01$). However, in contrast to the drop in frequency of sIPSCs, GM1 (1 μM) increased the average frequency of sEPSCs by 44.4 ± 19.2 % ($n = 10, P < 0.025$) from a control value of 0.36 ± 0.04 Hz ($n = 10$). These effects of GM1 on sEPSCs were also reversible with washing.

3.5.3.iii Effects of GM1 on isolated excitatory inputs to the pyramidal cells

To further isolate the spontaneous excitatory PSPs from the spontaneous inhibitory PSPs, we recorded (using 3 M KCl) from three CA1 cells in the presence of 10 μM BMI. TEA (10 mM) was then added to the ACSF in order to enhance the frequency and signal-to-noise ratio of the spontaneous EPSPs. The mean average frequency of the sEPSPs recorded in ACSF containing BMI and TEA (4.82 ± 2.06 Hz, $n = 3$) was approximately 13 fold greater than that recorded in normal ACSF (0.36 ± 0.04, $n = 10$), presumably owing to block of TEA-sensitive K^+- and $GABA_A$-gated Cl^--channels respectively, resulting in nerve terminal depolarization.

As illustrated in figure 3.6A, most of the spontaneous transients were sensitive to BMI while the remaining spontaneous PSPs were sensitive to KYN, and hence excitatory PSPs (data not shown). Under these conditions, 1 μM GM1 increased the frequency of sEPSPs by 17.4 ± 36.6 % from 4.82 ± 2.06 Hz ($n = 3$) ($P < 0.025$, Student's t-test for correlated groups). Furthemore, GM1 also significantly increased the incidence of large sEPSPs (cf. Figs. 3.7A and 3.7B, $\chi^2_{obt} = 60.6$, $P < 0.01$). This effect was observed in all three cells tested, and was reversible with washing (Fig. 3.7C).

3.5.4 Spontaneous 'miniature' PSCs (recorded in the presence of TTX)

3.5.4.i Effects of GM1 on the miniature EPSCs in CA3 cells

Our evidence thus far suggests that GM1 may increase excitatory inputs to CA1 neurons via an enhanced presynaptic firing. However, in order to assess whether GM1 can directly modulate transmitter release in the absence of presynaptic cell firing, we recorded

spontaneous PSCs while superfusing the slices with TTX. Due to the limitations of the intracellular recording technique and the distant location of most excitatory synapses (mentioned earlier), in agreement with previous observers (Ropert et al, 1990), we could not distinguish spontaneous EPSPs in the three CA1 cells recorded in the presence of TTX (data not shown). Nevertheless, spontaneous miniature EPSCs have been reported in CA1 neurons by other investigators using the whole cell patch clamp technique, which substantially improves the signal-to-noise ratio (Bekkers et al., 1990; Manabe et al., 1992).

Therefore we recorded kynurenate-sensitive spontaneous miniature excitatory PSPs from five CA3 cells, where the mossy fibres terminate close to the soma - thus circumventing most of the technical difficulties associated with the 'sharp electrode' voltage-clamp technique (Johnston and Brown, 1983). In addition, TTX blocked action potential-dependent transmitter release and BMI blocked all $GABA_A$ receptor-mediated IPSCs.

As illustrated in figure 3.8A, GM1 increased the frequency of the miniature EPSCs by 25.3 $\pm$ 10.6% (n = 5, from a mean control value of 3.7 $\pm$ 1.5 Hz, $P < 0.05$, one tail paired t-test). There was only a slight but statistically significant increase in the probability of larger spontaneous miniature EPSCs (cf. Figs. 3.8B and 3.8C, $\chi^2_{obt} = 17.7$, $P < 0.01$). Therefore, the data suggest that in addition to the facilitation of on-going excitatory inputs via an augmentation in presynaptic cell firing, GM1 can also enhance spontaneous transmitter release from the nerve terminals.

3.5.4.ii Effects of GM1 on the miniature IPSCs in CA1 cells

We also recorded spontaneous miniature IPSCs from five CA1 neurons in the presence of TTX. In agreement with the observations on electrically evoked monosynaptic IPSPs, GM1 did not increase either the frequency (0.39 ± 0.07 Hz in control and 0.40 ± 0.04 Hz in GM1, $n = 7$) or the amplitude of the spontaneous miniature IPSCs (cf. Figs. 3.9B and 3.9C). Therefore, these studies further suggest that the GM1-induced depression of the inhibitory inputs to CA1 cells is mediated indirectly.

3.6 Discussion

Previous findings have shown that exogenous monosialoganglioside (GM1) potentiates the induction of LTP in hippocampal slices (Wieraszko and Seifert, 1985; Ramirez et al., 1990). However, the mechanism of GM1's action on synaptic transmission is still not well defined. Hence the main goal of this study was to understand the modulatory role of exogenous GM1 in synaptic transmission between CA3 and CA1 pyramidal cells. Our results demonstrate that GM1 potentiates excitatory synaptic transmission, and suppresses inhibitory inputs, though without affecting directly the inhibitory terminals.

3.6.1 The effects of GM1 on excitatory synaptic transmission

3.6.1.i Increasing presynaptic firing

In extracellular recordings from the CA1 region, GM1 increases EPSPs and population spike evoked by orthodromic (Schaffer collateral) stimulation. At the same time, the afferent volley:EPSP (input:output) relation becomes steeper, but there is no change in the coupling between EPSPs and population spikes. These observations suggest that the enhancement of the population spikes is due to potentiation of excitatory synapses. The enhanced population spike amplitude may, in addition, reflect the ability of GM1 to recruit neighbouring cells via collateral branching (Andersen, 1975; Christian and Dudek, 1988).

Intracellular recordings showed that GM1 causes membrane depolarization and subsequently induces spontaneous firing. The enhanced cell firing may have resulted partly from a reduction in spontaneous IPSPs and an increase in spontaneous EPSPs (discussed below). These effects might therefore be expected to enhance the slope of the population spike versus EPSP plot (or E/S coupling); however, the failure to observe any change in E/S coupling (Fig. 3.1B) may have been caused by spike inactivation following the initial membrane depolarization.

The increased amplitude of the afferent volleys recorded in stratum radiatum may be explained if the enhanced activity of CA3 neurons increases the afferent fibres' excitability. Alternatively, GM1 may act directly on the Schaffer collaterals, thereby increasing their excitability.

The most convincing evidence indicating a possible increase in CA3 cell activity by GM1, however, was obtained from studies on spontaneous EPSPs or EPSCs recorded

in CA1 cells. In these, GM1 consistently increased both the frequency and amplitude of sEPSPs or sEPSCs. Furthermore, the rightward shift in the amplitude distribution histogram, observed in all the cells tested, can be interpreted as due to enhanced synchronous CA3 cell firing, resulting in a higher probability of simultaneous firing of Schaffer collateral fibres and therefore larger sEPSPs (Andersen and Lømo, 1966; Masukawa and Prince, 1984). Indeed, cutting the connections between CA3 and CA1 greatly reduced these spontaneous excitatory signals thus confirming that they are generated by the firing of CA3 cells.

3.6.1.ii Increasing transmitter release

The increased slope of the EPSP amplitude versus afferent volley (input:output)plot (Fig. 3.2B) indicates that during GM1 perfusion, there could be an enhanced excitatory transmitter release or an increase in postsynaptic sensitivity to glutamate (the presumed transmitter). The former is supported by intracellular recordings of TTX-insensitive spontaneous excitatory PSPs in which the frequency is consistently augmented by GM1. Thus in view of these findings, GM1 facilitates transmitter release via a combination of action potential-dependent and -independent pathways. This also suggests possible interactions between GM1 and membrane proteins affiliated with the release mechanism.

Indeed, due to the high concentration of GM1 located in the outer lipid layer of the nerve terminals (Fuxe et al., 1989) and the Ca^{2+} binding properties of gangliosides (McDaniel and McLaughlin, 1985), numerous investigators have postulated that under

resting conditions, GM1 strongly binds extracellular Ca^{2+} near the nerve terminals. When an action potential invades the nerve terminal thereby inducing membrane depolarization, the affinity of GM1 for the bound Ca^{2+} may diminish, thus liberating Ca^{2+} in the vicinity of presynaptic Ca^{2+} channels (Rahmann et al., 1982; Wolf and Irwin, 1991). In addition to the action potential-dependent release, GM1 has also been shown to activate transmembrane second messengers, in particular IP_3, thereby elevating intracellular Ca^{2+} via an internal storage pool and consequently facilitating transmitter release (Skaper et al., 1987; Leray et al., 1988). The lack of any GM1 effect on the frequency of TTX-insensitive spontaneous IPSCs is in agreement with previous reports which showed that GM1 may modulate GABA release by a different mechanism (Frieder and Rapport, 1981 & 1987).

3.6.1.iii Postsynaptic ligand-receptor interaction

In addition to the increased transmitter release, GM1 may enhance the postsynaptic receptor sensitivity to its ligand, as suggested by the short-lasting increase in the postsynaptic glutamate current. This observation is consistent with previous findings that GM1 stimulates glutamate binding in the hippocampus and cortex (Hollmann and Seifert, 1986). However, the mechanism by which GM1 potentiates the interaction between glutamate and glutamate receptors is still unclear (Hollmann and Seifert, 1989).

In the present study, we do not have any direct evidence suggesting that the gradual fading of glutamate current is due to receptor desensitization. Nevertheless, augmentation

of excitatory transmitter release by GM1 may contribute to a gradual fading of the iontophoretic glutamate current.

3.6.2 The effects of GM1 on inhibitory synaptic transmission

A disinhibitory action was observed in slices pre-incubated with GM1. After prolonged GM1 exposure, slices often generate epileptiform activity, manifested as multiple spiking. This finding is in agreement with whole animal studies which showed that excessive accumulation of GM1 induces epilepsy (Seyfried et al., 1978). However, administration of antibodies against GM1 (Kopeloff et al., 1942; Karpiak et al., 1981), and blockade of ganglioside receptors (Karpiak et al., 1978*b*) have also been shown to produce epilepsy, although these findings are still controversial (Seyfried et al., 1981).

The induction of multiple spikes in the stratum pyramidale by GM1 is in keeping with the intracellular data showing that GM1 is selective in its opposite actions on the excitatory (as discussed above) and inhibitory inputs to the CA1 neurons. The reduction in the frequency of bicuculline-sensitive spontaneous IPSCs suggests that GABA release is suppressed by GM1. This hypothesis appears to be further supported by the reduction in the slope of the voltage-current and current-voltage plots of the evoked inhibitory responses. Hence these observations would be in keeping with earlier suggestions that endogenous gangliosides have an on-going depressant effect on depolarization-evoked GABA release (Frieder and Rapport, 1987).

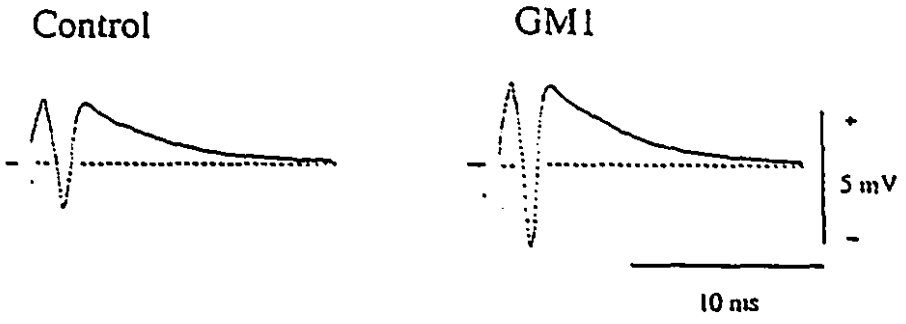
However, our observations on evoked monosynaptic IPSPs and TTX-insensitive

spontaneous miniature IPSCs show that GM1 has no direct effect on the inhibitory synapses. Therefore it is likely that the reduction in inhibitory inputs to CA1 by GM1 is an indirect effect of diminished excitability or synaptic activation of inhibitory cells. The former seems more likely because GM1-induced facilitation of excitatory inputs would be expected to enhance the firing of these cells via feed-forward (Alger and Nicoll, 1982) and recurrent pathways (Kandel et al., 1961; Andersen et al., 1964*a* and 1964*b*).

3.7 Figures

Figure 3.1. GM1 enhanced the positive wave (EPSP) and population spike, but did not alter the population spike-to-EPSP relation for Schaffer collateral-commissural evoked responses in the stratum pyramidale of rat hippocampal slice. *A* shows evoked response recorded from the pyramidal layer following sub-maximal orthodromic stimulation of the Schaffer collaterals. The initial positive wave is the field EPSP, which is followed by a large negative wave representing synchronous firing of the CA1 pyramidal cells. Superfusion with GM1 (1 μM) for 10 to 15 min enhanced the amplitudes of both the positive wave and population spike. In *B*, the slice was stimulated with intensities ranging from subthreshold to maximal. The amplitude of population spike was plotted as a function of the slope (dV/dt) of the positive wave (E/S coupling). A second order regression fit was used to plot the input-output curve before (open circles) and during GM1 perfusion (1 μM, filled circles). Using identical intensity range as in control, GM1 elevated the maximum population spike amplitude but did not change the input-output curve of population spike to EPSP relation.

A


Control
GM1
+
5 mV
-
10 ms

B

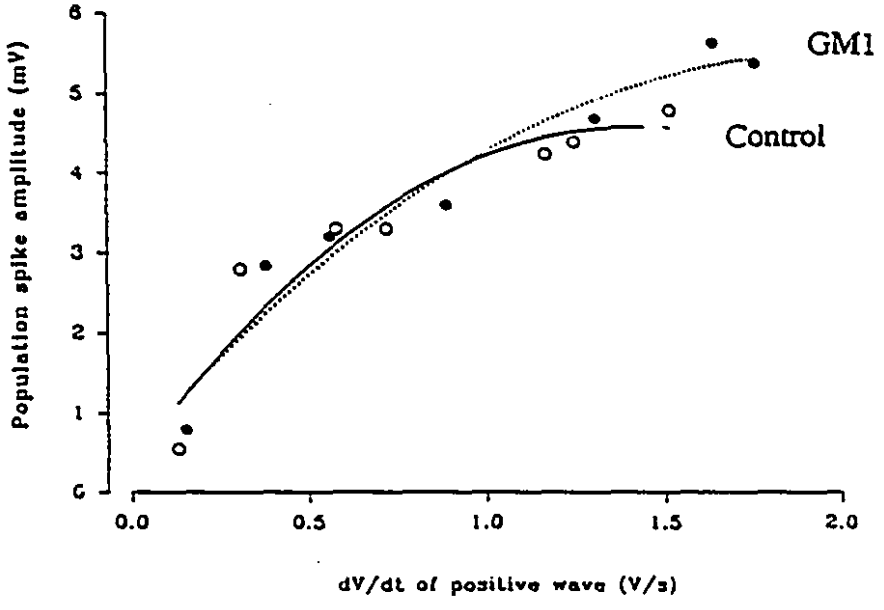

GM1
Control
Population spike amplitude (mV)
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
dV/dt of positive wave (V/s)

Figure 3.2. GM1 augmented the slope of the input-output curve (field EPSP to afferent volley relation) for Schaffer collateral-commissural evoked responses in the stratum radiatum. The stimulus intensities, ranging from subthreshold to maximal, were identical before (open circles) and during 1 μM GM1 perfusion (filled circles). The EPSP field amplitude was plotted as a function of the afferent volley, and subsequently fitted by a first order linear regression. The insert illustrates dendritic field potential elicited by orthodromic stimulation of the Schaffer collaterals. The first (and smaller) negative wave represents the afferent volley, which is followed by a larger negative wave representing the dendritic EPSP. GM1 (1 μM) increased both the EPSP amplitude and afferent volley. The stimulation intensity was identical for both traces and initially set at 50% of maximum.

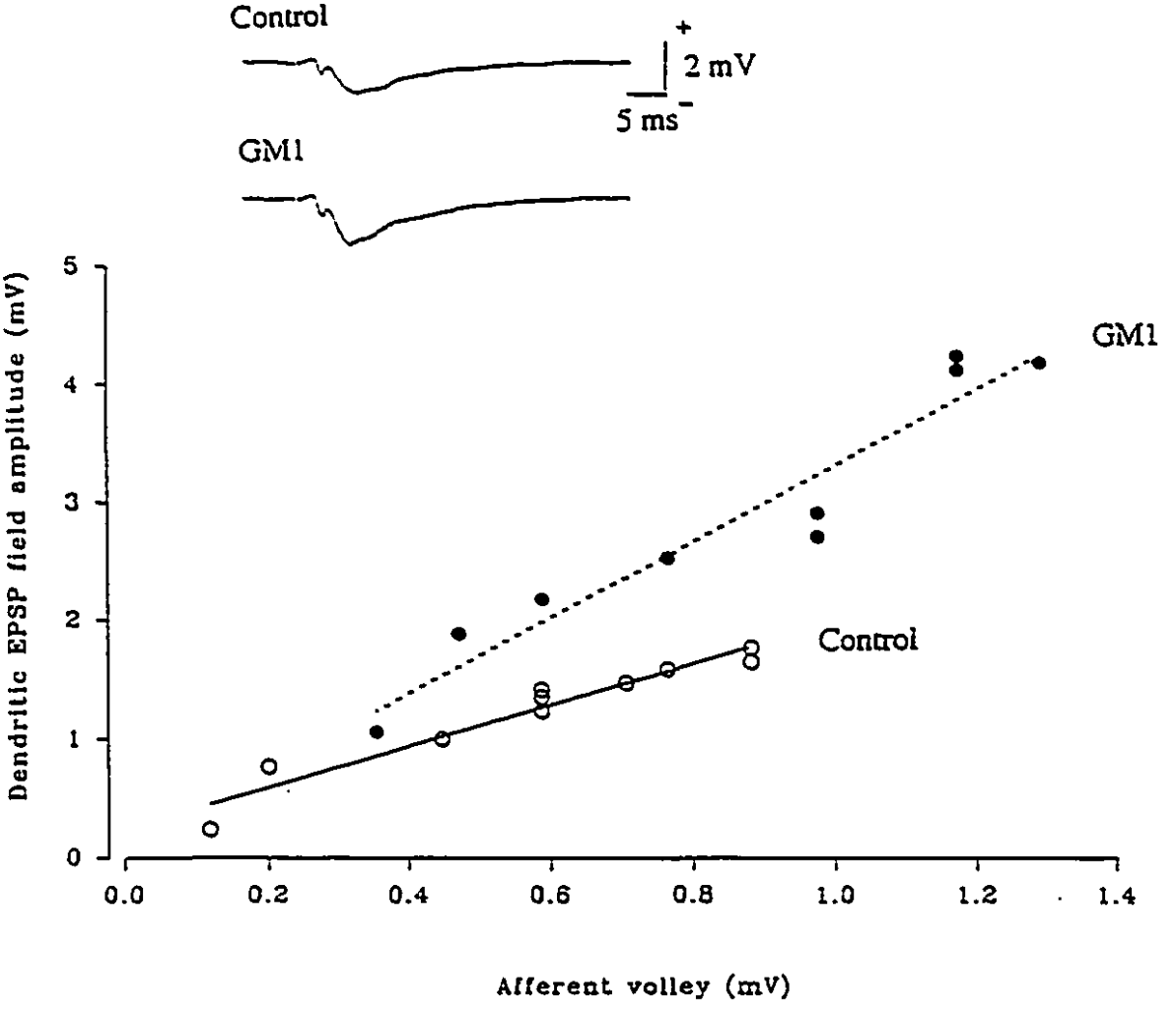
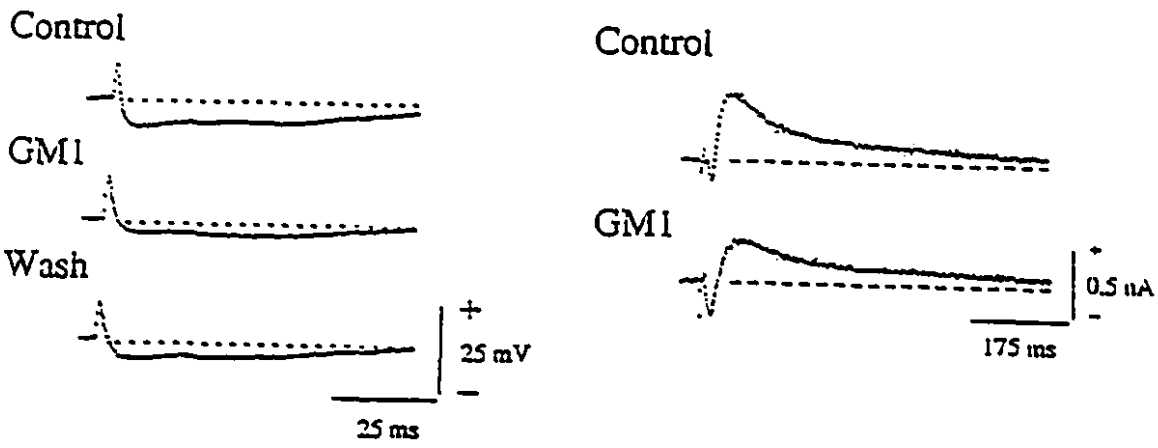

Control
+
2 mV
5 ms
−
GM1
Dendritic EPSP field amplitude (mV)
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
GM1
Control
Afferent volley (mV)

Figure 3.3. GM1 selectively enhances evoked EPSP while suppressing evoked IPSPs recorded in CA1 pyramidal cells. Synaptic transients were evoked by orthodromic stimulation (subthreshold for post-synaptic spikes) of the Schaffer collaterals. During current clamp recording (*A*), GM1 (1 μM) increased the amplitude of EPSP while reducing the amplitudes of fast and slow IPSPs. Initial resting membrane potential for this cell was -70 mV, and was adjusted to control level before taking measurements during GM1 superfusion and wash. Similar effects were observed in another cell under voltage-clamp, as shown in *B*. The holding potential was -60 mV. *C* shows a current-voltage (I/V) relation of IPSC evoked by orthodromic stimulation of the Schaffer collaterals. GM1 (1 μM, filled circles) reduced the peak amplitudes of early IPSCs measured over a wide range of holding potentials. Each point represents an average of 5 individual IPSC ($\pm$ S.E.). The graph was fitted by linear regression to control data (open circles; dotted line) and to data in the presence of GM1 (filled circles; solid line).

A


Control
GM1
Wash
+
25 mV
−
25 ms
Control
GM1
+
0.5 nA
−
175 ms

B

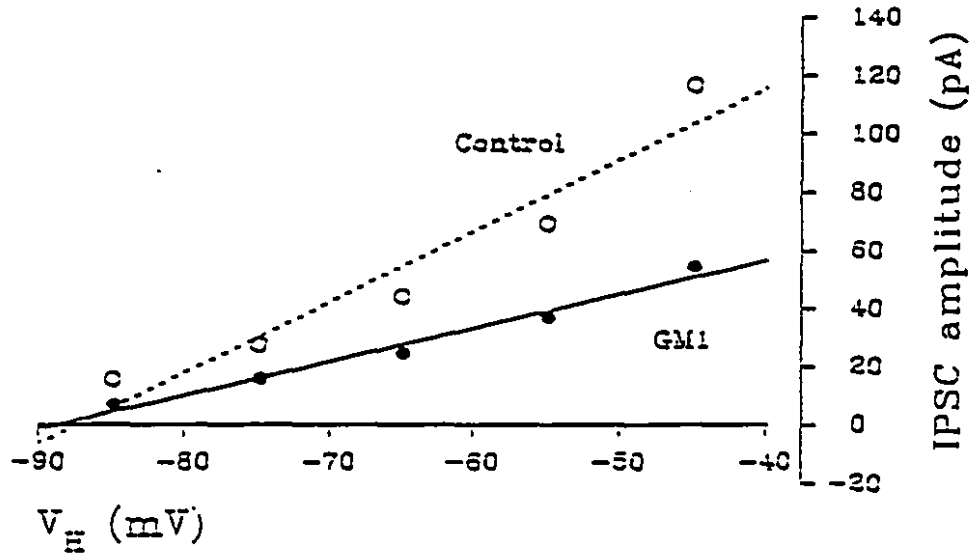
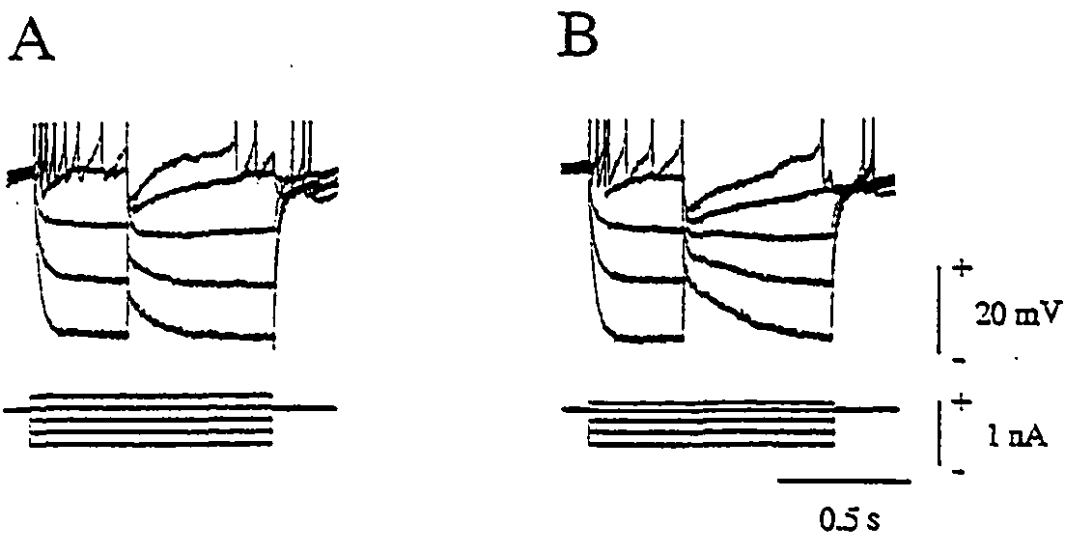

Control
GM1
140
120
100
80
60
40
20
0
-20
IPSC amplitude (pA)
-90
-80
-70
-60
-50
-40
V_H (mV)

Figure 3.4. GM1 did not alter monosynaptically-evokedIPSPs, and had little effect on the voltage-sensitivity of monosynaptic IPSPs. *A* shows voltage responses of a CA1 neuron (top traces) to current injections (bottom traces), and electrical stimulation (arrow) of the Schaffer collaterals, subthreshold for post-synaptic spike. Monosynaptic IPSPs were evoked by stimulating the stratum radiatum (close to the recorded pyramidal cell) in the presence of kynurenic acid (2 mM) - to remove excitatory inputs - and Cs^+ (4 mM), to block the voltage-dependent sag. The initial resting membrane potential was -70 mV. Under these condition, GM1 (1 μM, *B*) did not reduce the voltage-sensitive monosynaptic IPSP amplitudes. The spikes seen in *A* and *B* are truncated. With continuous superfusion of kynurenic acid, the voltage-current relation for the monosynaptic IPSPs (*C*) shows a substantial rectification for membrane potentials more negative than -80 mV (open circles). Cs^+ (4 mM) was then added to the perfusate in order to get a clear reversal of the monosynaptic IPSPs (open triangles); and these values were used as control for the subsequent GM1 perfusion (filled circles). Filled triangles represent wash with ACSF containing kynurenic acid and Cs^+.


A
B
20 mV
1 nA
0.5 s

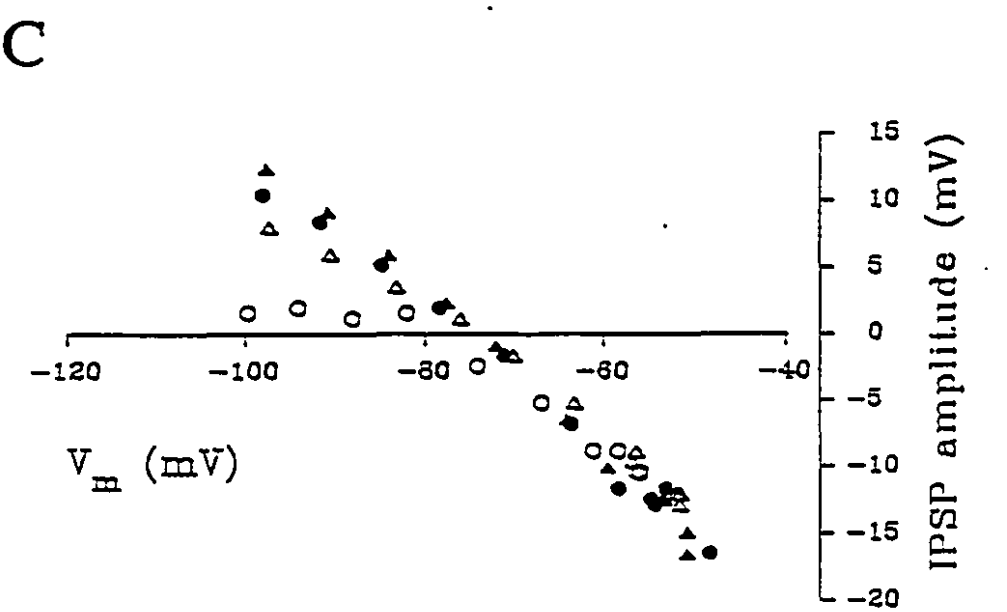
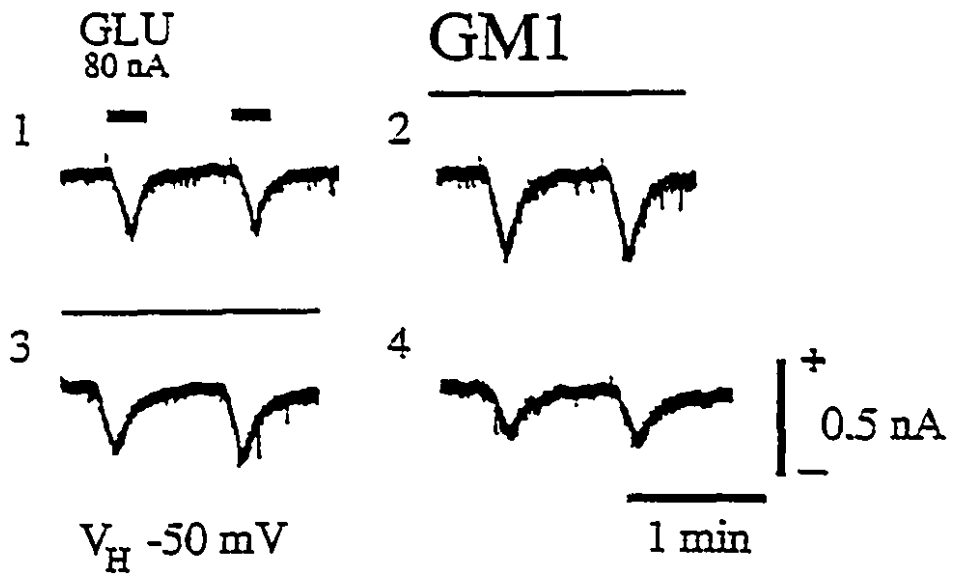

C
15
10
5
0
-5
-10
-15
-20
IPSP amplitude (mV)
-120
-100
-80
-60
-40
V_m (mV)

Figure 3.5. GM1 transiently potentiates inward currents elicited by glutamate applications (*A*). Brief pulses of L-glutamate were applied iontophoretically to the stratum radiatum in the presence of TTX while recording from a CA1 cell with a KCl-electrode (1 μM, *A1*). Pulse frequency was usually set at 0.02 Hz, and current intensity was initially adjusted to elicit half maximal glutamate-evoked currents. The spontaneous transient inward currents were kynurenate-insensitive but could be abolished by 10 μM bicuculline. In this cell, prolonged GM1 perfusion induced a gradual 'fading' of the glutamate evoked current (*A3* and *A4*). A total of 12 CA1 cells was studied, and *B* summarizes the time course of GM1 action on the glutamate-evoked current. The GM1-induced augmentation peaked after 4 min of GM1 perfusion. Each column represents the mean of 12 cells $\pm$ S.E.M.

A


GLU
80 nA
GM1
1
2
3
4
+
0.5 nA
−
V_H -50 mV
1 min

B

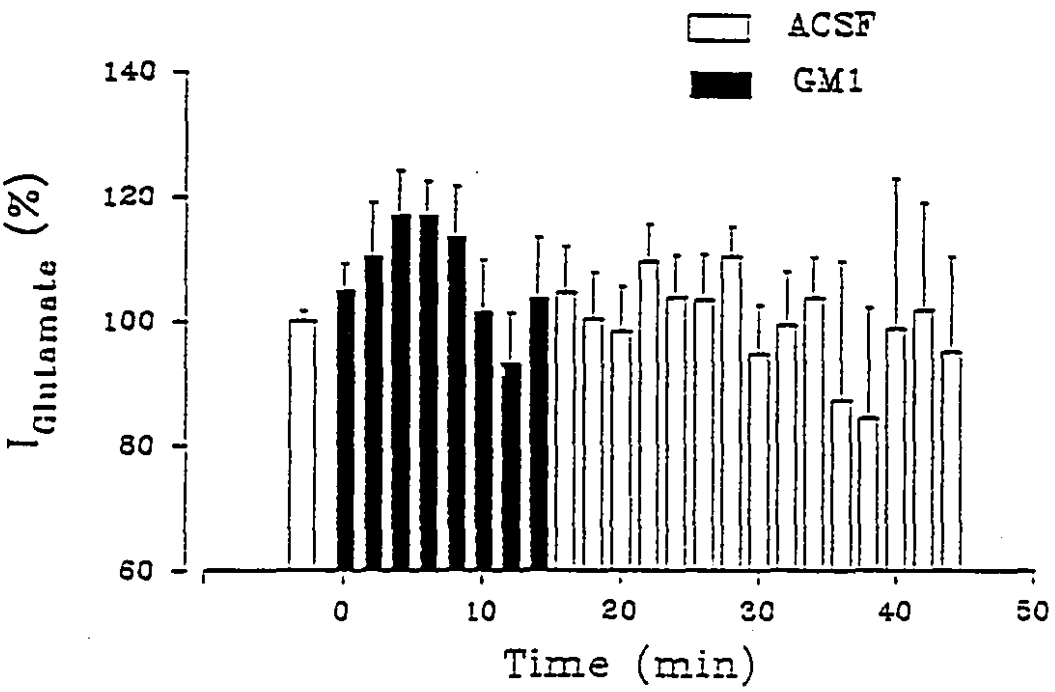

ACSF
GM1
140
120
100
80
60
$I_{Glutamate}$ (%)
0
10
20
30
40
50
Time (min)

Figure 3.6. In the absence of $GABA_A$ receptor-mediated IPSPs, GM1 enhances the spontaneous TTX- and kynurenate-sensitive EPSPs recorded from a CA1 pyramidal cell. In *A*, most of the spontaneous transient depolarizations were blocked by 10 μM bicuculline (BMI). Tetraethylammonium chloride (TEA, 10 mM) was then added to the perfusate to enhance the spontaneous EPSPs. In another cell (*B*), both the frequency and amplitude of the spontaneous EPSPs were enhanced by GM1 (1 μM). The control solution for this cell contained BMI and TEA as in *A*.

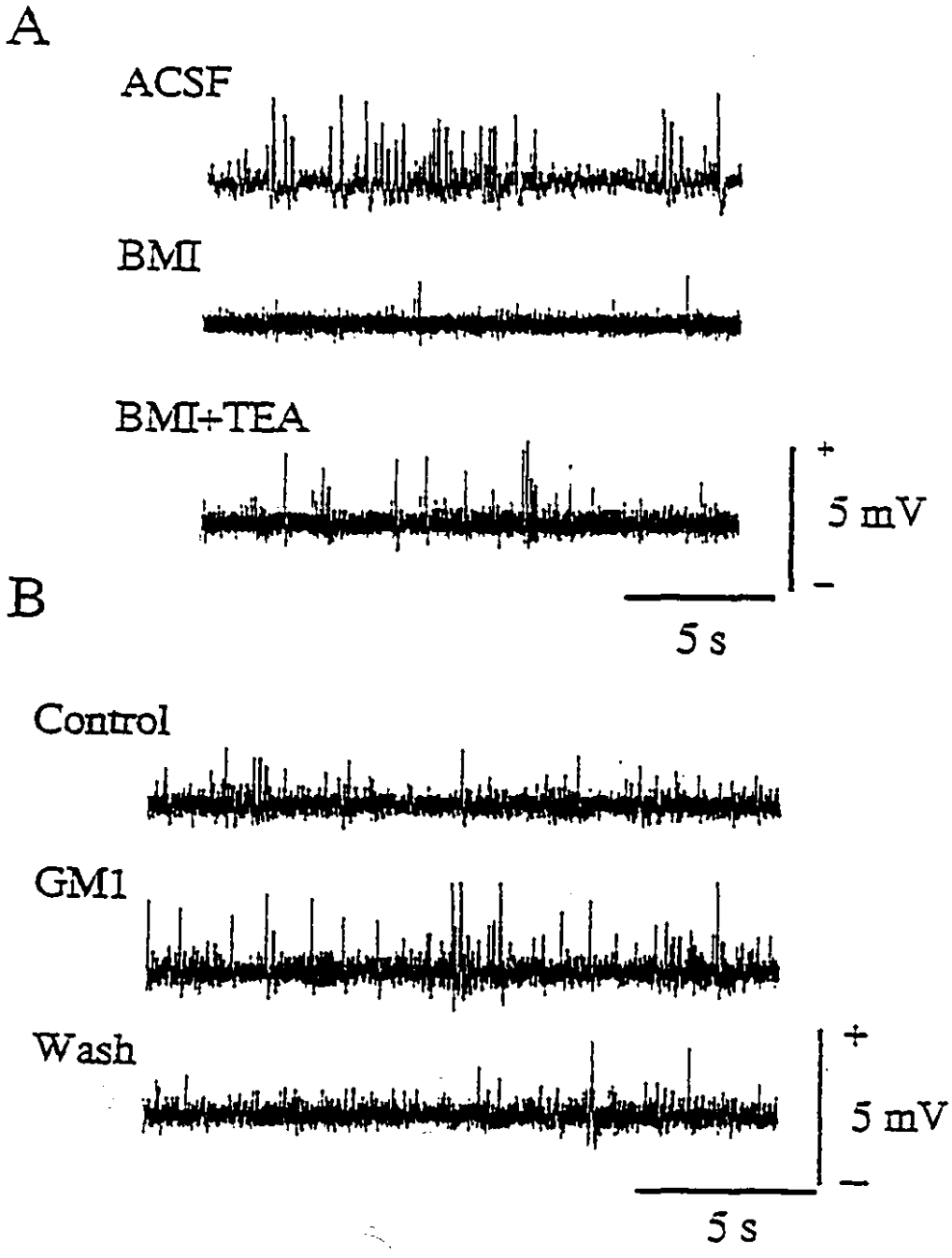

A
ACSF
BMI
BMI+TEA
+
5 mV
−
5 s
B
Control
GM1
Wash
+
5 mV
−
5 s

Figure 3.7. GM1 increases both the frequency and amplitude of spontaneous TTX- and kynurenate-sensitive EPSPs recorded from a CA1 pyramidal cell. Amplitude distribution histograms of the spontaneous EPSPs were measured in control ACSF (*A*), GM1 (1 μM, *B*), and after wash (*C*). Throughout the experiment, BMI and TEA were present in the perfusate. Note that in *B*, GM1 induced a higher incidence of larger spontaneous EPSPs. *n* represents the total number of spontaneous EPSPs counted during 22 s periods.

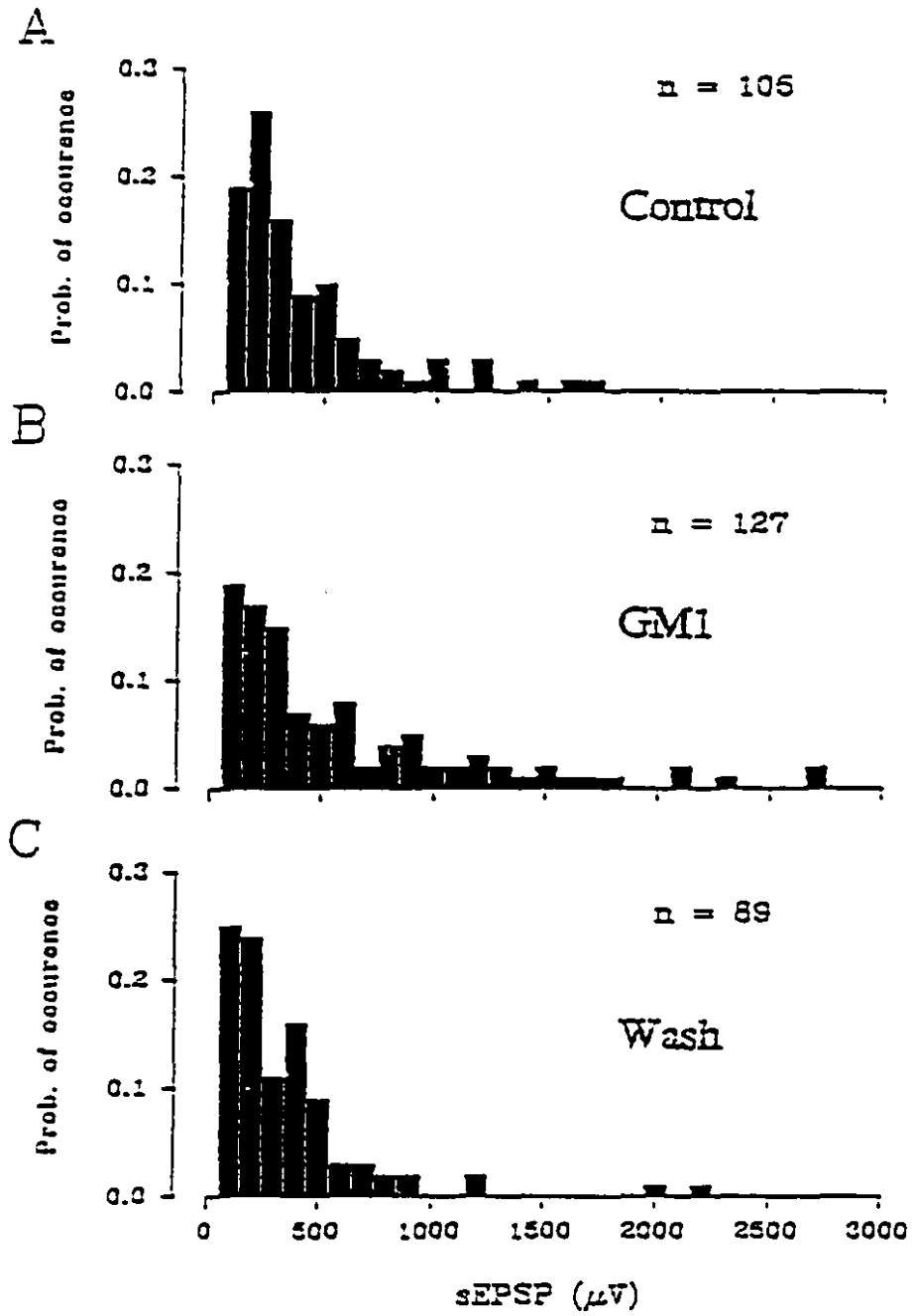

A
B
C
n = 106
Control
n = 127
GM1
n = 89
Wash
Prob. of occurence
0.3
0.2
0.1
0.0
0
500
1000
1500
2000
2500
3000
sEPSP (μV)

Figure 3.8. GM1 increases the frequency but not the amplitude of the spontaneous TTX-insensitive miniature EPSCs recorded from CA3 cells. The control solution (*A*) contains BMI (10 μM) to block all $GABA_A$-mediated IPSCs. *B* and *C* are amplitude distribution histograms of the spontaneous EPSCs recorded in the presence of TTX in control solution and GM1 (1 μM) respectively. *n* represents the total number of miniature EPSCs counted in 3.5 min periods. GM1 (*C*) did not change the amplitude distribution of miniature EPSCs.

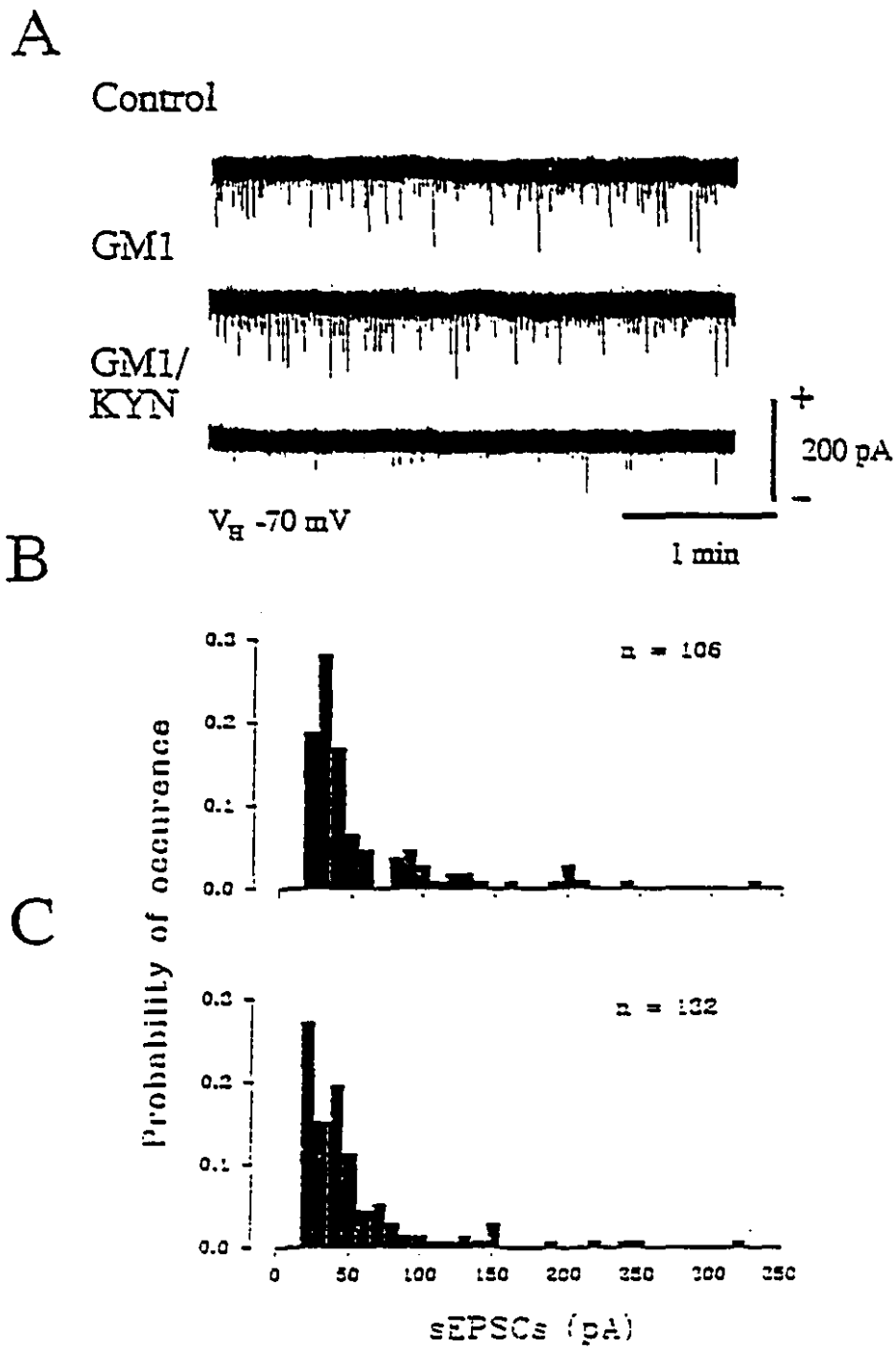
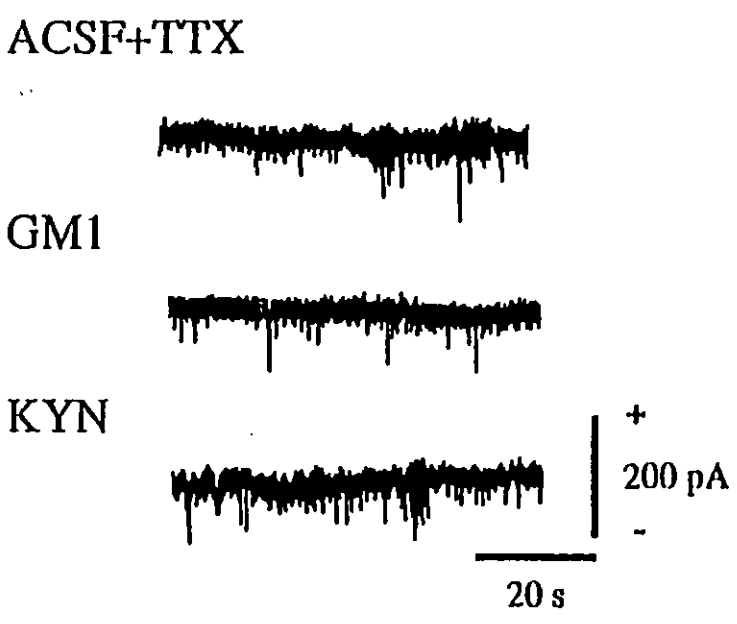

A
Control
GM1
GM1/
KYN
+
200 pA
−
V_H -70 mV
1 min
B
C
n = 106
n = 132
Probability of occurrence
0.3
0.2
0.1
0.0
0
50
100
150
200
250
300
350
sEPSCs (pA)

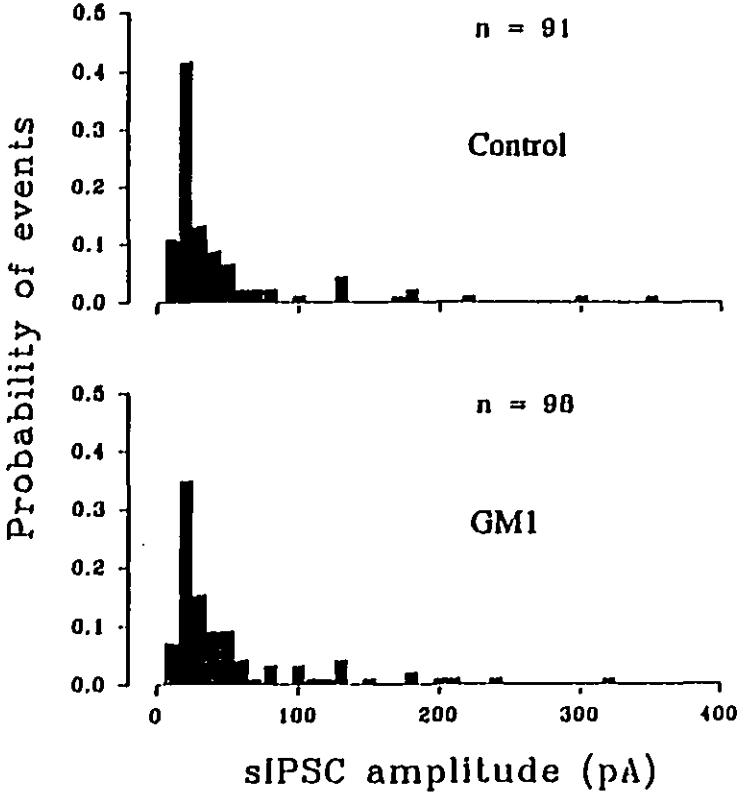
Figure 3.9. GM1 had no effect on the frequency or the amplitude of the spontaneous miniature IPSCs recorded from CA1 cells in the presence of TTX. *A* shows current traces containing spontaneous IPSCs of a CA1 cell recorded in the presence of GM1 (1 μM) and subsequently in kynurenic acid (KYN, 2 mM). This cell was impaled with a micropipette containing 3 M KCl; and the holding potential was at -65 mV. Shown in *B* are amplitude distribution histograms obtained in control ACSF (containing 1 μM TTX) and in presence of GM1 (1 μM) respectively. *n* represents the total number of miniature IPSCs counted in 1 min periods.

A


ACSF+TTX
GM1
KYN
+
200 pA
-
20 s

B

C


n = 91
Control
n = 98
GM1
Probability of events
0.0
0.1
0.2
0.3
0.4
0.5
0
100
200
300
400
sIPSC amplitude (pA)

3.8 References

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CHAPTER 4

4.1 MODULATION OF HIGH VOLTAGE ACTIVATED Ca^{2+} CURRENTS BY GANGLIOSIDE GM1

P. Miu and K. Krnjević

To be submitted

4.2 Summary

1. Previous studies have shown that high voltage activated (HVA) calcium currents (N/L types) initiate Ca^{2+}-dependent transmitter release, and that the monosialoganglioside (GM1) blocks the protein kinase C-mediated potentiation of HVA Ca^{2+} currents. However, GM1 has also been shown to selectively potentiate spontaneous glutamate release. This paradoxical action of GM1 subsequently prompted us to further examine the interaction between GM1 and HVA Ca^{2+} currents, using the single-electrode voltage-clamp (SEVC) technique in CA1 pyramidal neurons of rat hippocampal slices.
2. Sharp intracellular micropipettes containing either 3 M CsCl or 3 M Cs acetate were used to impale fifty-eight CA1 pyramidal neurons. To isolate HVA Ca^{2+} currents, inward Na^+ current was blocked by tetrodotoxin (1 μM; TTX) and the outward K^+ currents were blocked by tetraethylammonium chloride (10 mM, TEA), CsCl (4 mM), and 4-aminopyridine (100 μM, 4AP). The holding membrane potentials (V_H) were -50 to -40 mV.
3. HVA inward currents (showing an initial transient peak and then a more slowly decaying 'steady state' component) were elicited by positive voltage commands. Both peak and 'steady state' inward currents were suppressed by Mn^{2+} (2.3 mM) and low Ca^{2+} (0.4 mM) thus confirming their Ca^{2+} dependence.

4. GM1 (1 μM) reduced the peak current by 15.6 $\pm$ 4.2 % (n = 32, $P < 0.001$), and the 'steady state' current less consistently by 11.1 $\pm$ 5.6 % (n = 29, $P \approx 0.05$). The GM1-induced reduction of HVA Ca^{2+} currents was blocked by kynurenic acid (1 mM), but not by bicuculline (10 μM).

5. In cells loaded with chloride (recording with CsCl-filled electrodes), GM1 (1 μM) consistently induced an inward shift in the holding current and an increase in the leak conductance. The inward holding current is probably mediated by a Ca^{2+}-dependent mechanism since it was blocked by Mn^{2+} and low Ca^{2+}. Furthermore, superfusion of kynurenic acid (1 mM) was also effective in preventing the GM1-induced inward holding current and the reduction in the leak conductance. Bicuculline (10 μM) had no effect on the GM1-induced inward holding current but blocked the increase in leak conductance.

6. In cells *not* loaded with chloride (recording with Cs acetate-filled electrodes), GM1 (1 μM) induced a slight outward holding current and a reduction in the leak conductance.

7. These findings suggest that the GM1-induced inward shift in the holding current is mediated by a glutamate dependent Ca^{2+} influx. The potentiated glutamate release would increase intraneuronal Ca^{2+} concentration, thereby triggering Ca^{2+}-dependent Ca^{2+} inactivation. Elevation of intracellular Ca^{2+} concentration could therefore explain the reduction in HVA Ca^{2+} currents. In addition, activation of chloride conductance by GM1 also played a significant role in reducing HVA Ca^{2+} currents.

4.3 Introduction

Intracellular free calcium ($[Ca^{2+}]_i$) is at a very low concentration in all cells. In mammalian neurons, changes in $[Ca^{2+}]_i$ have been shown to regulate various cellular responses. These responses include a wide range of diverse functions such as transmitter release (Katz, 1969; Llinas et al, 1976; Baker and Knight, 1978; Baker et al, 1980), Ca^{2+}-dependent ionic conductances, and hence cell excitability (Krnjević et al, 1975; Alger and Nicoll, 1980; Benardo and Prince, 1982; Owen et al, 1984; Owen et al, 1988), and Ca^{2+}-dependent activation of a variety of second messenger systems (Rasmussen and Barrett, 1984; Eberhard and Holz, 1988). The voltage-dependent low- and high-threshold Ca^{2+} channels, receptor operated Ca^{2+} channels, and internal Ca^{2+} storage pools are the main mechanisms known so far to induce both transient and sustained accumulation in $[Ca^{2+}]_i$ (for a detailed review see Tsien and Tsien, 1990).

Three different voltage-dependent Ca^{2+} channels, classified as T- (low voltage-activated; LVA) and N/L-types (high-voltage activated; HVA), have been identified in hippocampal pyramidal CA1 neurons (Brown et al, 1990; Fisher et al, 1990). Although HVA Ca^{2+} channels demonstrate voltage-dependent activation and inactivation, other findings have shown that phosphorylation or dephosphorylation of HVA Ca^{2+} channel subunits by second messengers, in particular protein kinase C (PKC), can modify channel opening or closing kinetics (Fedulova et al, 1981; DeRiemer et al, 1985; Chad and Eckert, 1986; Toselli et al, 1989; Trautwein and Hescheler, 1990). Moreover, sphingosine,

derived from metabolic breakdown of membrane sphingolipids or newly incorporated exogenous ganglioside GM1, has been shown to inhibit PKC activity, thus indirectly regulating HVA Ca^{2+} channels (Hannun et al, 1986; Kreutter et al, 1987; Hannun and Bell, 1989; Riboni et al, 1992).

Indeed, our earlier study on CA1 pyramidal neurons of rat hippocampal slices (Agopyan et al, 1992) has shown that HVA Ca^{2+} currents can be reduced by PKC antagonists such as GM1 and 1-(5-isoquinolinesulfonyl)-2-methyl piperazine (H-7). However, in addition to the antagonistic action of GM1 on PKC, we observed that GM1 alone could also reduce HVA Ca^{2+} currents. Since HVA Ca^{2+} currents initiate Ca^{2+}-dependent transmitter release (Perney et al, 1986; Rane et al, 1987; Hirning et al, 1988), the suppression of HVA Ca^{2+} currents by GM1 seems to contradict the hypothesis that GM1 modulates transmitter release (Burton and Howard, 1967; Rahmann et al, 1976; Miu and Krnjević, 1992). Therefore the main goal of this study was to explore the mechanisms underlying the GM1-mediated reduction of HVA inward Ca^{2+} currents, using the single-electrode voltage-clamp technique in hippocampal slices. Preliminary reports have been presented in abstract form (Agopyan et al, 1990; Miu and Krnjević, 1991*b* and 1991*c*)

4.4 Methods

4.4.1 Preparation of slices

Hippocampal slices were prepared and maintained by conventional techniques (Dingledine, 1984). Briefly, the brain of male Sprague-Dawley rats (150-200 g) was removed under halothane anaesthesia and immersed in pre-cooled (−4°) artificial cerebrospinal fluid (ACSF) that was oxygenated with 95/5% gaseous mixture of O_2/CO_2 respectively. The constituents of the ACSF were (in mM): NaCl 124, KCl 3, $MgCl_2$ 2, $CaCl_2$ 2, NaH_2PO_4 1.25, $NaHCO_3$ 26, and glucose 10. In low calcium experiments, $CaCl_2$ was reduced to 0.4 mM and $MnCl_2$ (2.3 mM) was added to further suppress Ca^{2+} currents.

After dissecting out the hippocampus, 450 μm thick transverse slices were cut with a McIlwain tissue chopper. Two to four slices were placed in an interface-recording chamber. The slices were superfused continuously at a constant rate of 2-3 ml/min with oxygenated ACSF at 33° (pH 7.4). The remaining slices were kept in the holding chamber and oxygenated with 95/5% O_2/CO_2 at room temperature. Slices were allowed to stabilize in the recording chamber for approximately one hour before electrical recordings.

4.4.1.i Recording techniques and arrangement

Conventional intracellular recording techniques were used in conjunction with an Axoclamp-2 amplifier (Axon Instruments Ltd.). A total of fifty-eight neurons of the CA1 pyramidal layer were impaled with single-barrel glass micropipettes containing either 3 M CsCl (R_e = 60-80 MΩ) or 3 M Cs acetate (pH 7.0, R_e = 90-120 MΩ). Only cells with stable resting potentials, input resistance (R_N) greater than 30 MΩ and action potentials greater than 85 mV were selected for GM1 administration.

The single electrode voltage-clamp (SEVC) technique was used to clamp thirty-nine CA1 cells impaled with CsCl-electrodes and nineteen cells impaled with Cs acetate-electrodes. The sampling frequency was usually set at 4-5 kHz, the clamp gain at 3-8 nA/mV, and the output bandwidth at 0.3-1 kHz. The input waveform was continuously monitored to ensure that capacity neutralization was near optimal.

To isolate HVA Ca^{2+} currents, inward Na^{+} currents were blocked by 1 μM TTX, and most of the outward K^{+} currents were blocked by tetraethylammonium chloride (10 mM, TEA), CsCl (4 mM), and 4-aminopyridine (100 μM, 4AP). The holding potentials ranged between -50 and -40 mV. HVA Ca^{2+} currents were induced by either 200 ms or 400 ms depolarizing voltage commands at 0.05 Hz. Leak currents were assessed by applying hyperpolarizing commands. The net peak and 'steady state' (measured at the end of either 200 or 400 ms pulses) inward currents were values obtained after subtracting the extrapolated leak currents.

The voltage clamp data were monitored continuously on a Gould pen recorder and on an oscilloscope. Traces were stored on an IBM AT computer for subsequent off-line analysis. All values are reported as means $\pm$ standard errors. Statistical analysis were performed on all the data by paired Student's t-test, unless indicated otherwise. A significance level of $p < 0.05$ was chosen for all statistical comparisons.

4.4.1.ii Solutions

Tetrodotoxin (TTX), tetraethylammonium chloride (TEA), 4-aminopyridine (4AP),

CsCl, and (-)-bicuculline methiodide (BMI), all from Sigma Inc., were dissolved in distilled water and diluted to their final concentration in the perfusing medium. Kynurenic acid (Sigma Inc.) was added directly to the ACSF.

Monosialoganglioside GM1 (M.Wt. 1569) was dissolved in distilled water and further diluted to a final concentration of 1 μM in the perfusing medium. GM1 was a gift from Dr. Toffano at FIDIA Institute (Abano Terme, Italy).

4.5 Results

4.5.1 High voltage activated (HVA) inward Ca^{2+} currents

In the presence of Na^+ and K^+ channel blockers and with a holding potential (V_H) of -50 or -40 mV, HVA currents were evoked by depolarizing voltage commands which lasted either 200 or 400 ms. The inward current response consisted of an initial transient and a final 'steady state' component (Figs. 4.1, 4.2 and 4.4). These currents were mediated by Ca^{2+} influx since they were blocked by perfusing a Mn^{2+}- and low Ca^{2+}-containing solution (Fig. 4.2). In the present study, no further attempt was made to differentiate these calcium channels into either N or L type (cf. Brown et al, 1990; Fisher et al, 1990).

4.5.1.i Effects of GM1 recorded with CsCl-filled electrodes

In the control solution, the peak inward current was activated at potentials above

-40 mV, and reached a *maximum* amplitude near 0 mV (Fig. 4.1*B*). Under such recording conditions, GM1 (1 μM) reversibly reduced the peak and 'steady state' inward currents (Figs. 4.1*A*).

During GM1 perfusion, the peak inward current was reduced over a wide range of voltage, usually without any change in its voltage-dependence (including the threshold for activation). The current-voltage (I/V) relation for the net inward current, measured at the initial peak and corrected for the extrapolated leak current, before (open circles) and during GM1 perfusion (filled circles) is shown in figures 4.1*B*, 4.2*B*, and 4.4*B*. As summarized in figure 4.5, GM1 reduced the peak inward current significantly by 15.6 $\pm$ 4.2 % (n = 32; $P < 0.001$) and to a lesser degree the 'steady state' current, by 11.1 $\pm$ 5.6 % (n = 29, $P \approx 0.05$).

In addition, under the same condition of recording (including the presence of TTX), GM1 also frequently induced an inward shift in the holding current (I_H) and an increase in the leak conductance (G_L) (cf. Figs. 4.2*B*, 4.3*A* and 4.4*B*). Over the range of V_H between -65 and -35 mV, the GM1-induced inward I_H shift was by 82.8 $\pm$ 30.2 pA (n = 37, $P < 0.01$; Fig. 4.7), and the increase in G_L by 23.3 $\pm$ 5.0 % (n = 18, $P < 0.0005$; Fig. 4.6).

4.5.1.ii Effects of Mn^{2+} and low Ca^{2+}

While recording from eight CA1 pyramidal cells, a Mn^{2+} (2.3 mM) and low Ca^{2+} (0.4 mM)-containing solution was perfused in order to examine the mechanism of the

GM1-induced inward I_H and increase in G_L. Figure 4.3*A* shows the GM1-induced inward shift on a continuous chart record and its subsequent block by perfusate containing Mn^{2+} and low Ca^{2+} (see also I/V plot in Fig. 4.2*B*). Also visible in figure 4.3*A* are the prominent spontaneous inward currents which tend to appear during application of GM1 and are suppressed by Mn^{2+} and low Ca^{2+} perfusion.

In these eight cells, GM1 (1 μM) induced an inward shift by 221.3 $\pm$ 69.6 pA (n = 8; $P < 0.02$). In the presence of Mn^{2+} and low Ca^{2+}, this was reversed to a net outward shift, by 97.5 $\pm$ 39.9 pA (n = 8; $P < 0.05$). In addition, the net inward Ca^{2+} currents were virtually abolished in the Mn^{2+} and low Ca^{2+}-containing solution (Fig. 4.2*A*).

These findings suggest that the GM1-induced inward I_H is generated by an increase in some calcium-dependent process or ionic conductance(s). Since the inward Na^+ current was blocked by TTX, and these cells were chloride loaded, the likely ionic mechanisms involved include a slow inward Ca^{2+} current, an inward current carried by Cl^- efflux, an unspecific cationic conductance, and Na^+/Ca^{2+} exchange. An alternative possibility might be an indirect effect of a Ca^{2+}-dependent transmitter release. Hence, antagonists such as kynurenic acid and bicuculline were tested in order to eliminate any depolarizing effects generated by glutamate and GABA release.

4.5.1.iii Effects of kynurenic acid on GM1-mediated responses

It has been previously reported that GM1 enhances presynaptic glutamate release

(Miu and Krnjević, 1991 and 1992), and that subsequent activation of dendritic glutamate receptors induces Ca^{2+} influx resulting in a sustained increase in $[Ca^{2+}]_i$ (Regerhr and Tank, 1990; Wadman and Connor, 1992). Hence, we tested whether or not a wide spectrum glutamate antagonist kynurenic acid (Bertolino et al, 1989; Robinson et al, 1984) could prevent some of the GM1-induced changes, such as the inward shift in I_H and the attenuation of Ca^{2+} currents.

Applications of kynurenic acid alone produced a small, but statistically significant outward shift in I_H (by 24.3 $\pm$ 6.5 pA, n = 4; Fig. 4.6*A*) and no significant change in G_L (1.5 $\pm$ 4 %, n = 5; Fig. 4.6*B*). Figure 4.3*B* shows a continuous chart record from a CA1 pyramidal cell bathed in 1 mM kynurenic acid for approximately 10 min prior to GM1 perfusion. Under this condition, GM1 had no effect on I_H (3.5 $\pm$ 9.9 pA, n = 8) but significantly reduced G_L by 11.6 $\pm$ 3.7 % (n = 8, $P < 0.01$). These data are summarized in figure 4.6.

When the broad spectrum glutamate antagonist kynurenic acid was perfused alone in 5 tests, there was no significant change in the peak or 'steady state' inward Ca^{2+} currents (by 0.2 $\pm$ 11.9 % and -6.1 $\pm$ 3.6 % respectively). However, in all cells tested, and as summarized in figure 4.5, kynurenic acid prevented the GM1-induced reduction of the HVA Ca^{2+} currents: the peak currents changed non-significantly by 6.8 $\pm$ 11.6 % (n = 9) and the 'steady state' currents by -3.7 $\pm$ 3.3 % (n = 8; Fig. 4.4*A*). I/V plots of the same cell (Fig. 4.4*B*) show that the GM1-induced reductions in the peak inward Ca^{2+} currents were reversed by kynurenic acid over a wide voltage range. Also shown

in these I/V plots are the kynurenate-induced outward shift in I_H and a slight reduction in G_L.

4.5.1.iv Effects of bicuculline on GM1-mediated responses

Bath perfusion of the $GABA_A$ receptor antagonist bicuculline (BMI) alone induced an inward shift in I_H (by 51.7 ± 21.9 pA, $n = 11$, $P < 0.05$; Fig. 4.6*A*) without any significant change in G_L (0.4 ± 5.2 %, $n = 13$; Fig. 4.6*B*). However, in the presence of an ongoing BMI application (after stabilization of BMI-induced effects), GM1 still induced a net inward shift in I_H (46.8 ± 13.6 pA, $n = 5$, $P < 0.025$; Fig. 4.6*A*), but had no significant effect on G_L (reduction by 5.8 ± 3.7 %, $n = 5$; Fig. 4.6*B*).

Bicuculline alone enhanced the peak inward Ca^{2+} current by 11.9 ± 4.0 % ($n = 10$, $P < 0.02$; Fig. 4.5*A*) without any significant change in the 'steady state' current (reduction by 18.2 ± 12.2 %, $n = 10$; Fig. 4.5*B*). In spite of the significant increase in the peak inward currents produced by BMI, GM1 still did not significantly alter the peak inward currents (slight increase by 3.8 ± 3.7 %, $n = 12$) and 'steady state' inward currents (slight increase by 0.7 ± 5.6 %, $n = 11$; Fig. 4.5).

4.5.1.v Effects of GM1 with Cs acetate-filled electrodes

In order to assess whether Cl^- participated in the GM1-induced inward I_H, we recorded from nineteen cells with micropipettes filled with 3 M Cs acetate.

Figure 4.7 shows current-voltage relations for the GM1-induced changes in I_H,

observed repeatedly in chloride (circles) and non-chloride (triangles) loaded cells. Straight regression lines were fitted to the mean values of ΔI_H observed at various V_H's to show the different reversal potentials for the GM1 effects recorded with CsCl- and Cs acetate-filled electrodes: the calculated values for the reversal potential were -31 mV (from $\Delta I_H = 2.6\ V_H + 79.8$) and -62 mV (from $\Delta I_H = 1\ V_H + 61.6$) respectively.

In contrast to the chloride-loaded cells, individual results from acetate-loaded cells (for V_H ranging from -80 to -35 mV) indicated that GM1 produced no significant change in I_H (32.4 ± 27 pA, $n = 10$) but reduced G_L by 14.8 ± 6.6 % ($n = 8$, $P < 0.05$). Moreover, GM1 did not significantly reduce the peak or 'steady state' inward Ca^{2+} currents (by 18.3 ± 18.8 %, and 4.5 ± 12.9 % respectively, $n = 5$ for each mean).

4.6 Discussion

In agreement with our earlier findings (Agopyan et al, 1992), GM1 more consistently reduced the peak than the 'steady state' component of HVA Ca^{2+} currents in CA1 pyramidal neurons, recorded with CsCl-filled electrodes. However, in addition to the antagonistic effect of GM1 on protein kinase C-mediated augmentation of HVA Ca^{2+} currents (Agopyan et al, 1992), we now report that GM1 can also regulate HVA Ca^{2+} currents through a Ca^{2+}-dependent inward shift in the holding current. In this regard, we have isolated two probable ionic conductances underlying the GM1-induced inward shift in the holding current. These conductances include a Na^+/Ca^{2+} conductance, activated by

glutamate-gated channels, and a very small if at all significant inward Cl^- current, probably activated by a Ca^{2+}-dependent chloride conductance. Activation of these conductances may subsequently modulate HVA Ca^{2+} currents.

4.6.1 High voltage activated inward Ca^{2+} currents

In CA1 pyramidal neurons impaled with chloride containing electrodes, a consistent observation with GM1 perfusion, whether recorded in current- (Miu and Krnjević, 1992) or single electrode voltage-clamp mode, was a membrane depolarization, or inward shift in holding current. In addition, our findings suggest a possible link between GM1-induced inward shift in the holding current and the reduction in HVA Ca^{2+} currents.

The GM1-induced inward holding current is sensitive to Mn^{2+} and low Ca^{2+} containing solution thus suggesting the involvement of an inward Ca^{2+} current and/or a Ca^{2+}-dependent process or ionic conductance. Activation of these currents would undoubtedly augment the leak conductance as observed in cells recorded with CsCl-filled electrodes. However, an increased leak conductance alone does not adequately explain the suppression of the HVA Ca^{2+} currents, for the following reasons: 1) reductions in the leak conductance, as observed with cells impaled with Cs acetate-filled electrodes or application of GM1 over an on-going kynurenic acid perfusion, have no effect on the inward Ca^{2+} currents; 2) the 'steady state' inward Ca^{2+} currents were less consistently reduced; and 3) GM1 usually did not alter the voltage-dependence and the threshold for activation of the peak inward Ca^{2+} currents, as demonstrated by the I/V plots. Therefore, the GM1-induced reduction in HVA Ca^{2+} currents is unlikely to be due to a non-specific increase in leak

conductance.

4.6.2 Effects of glutamate on the holding current

Blockade of the GM1 effects by kynurenic acid, an antagonist of both non-NMDA and NMDA receptors, indicates the probable involvement of glutamate-mediated channel activation - in keeping with our previous evidence (Miu and Krnjević, 1992) that GM1 enhances ongoing glutamate release, as well as iontophoretic glutamate-evoked currents. Hence, increasing glutamate release in the synaptic cleft would activate both the NMDA and non-NMDA receptor-coupled channels resulting in Na^+ and Ca^{2+} influx. Any rise in intracellular Na^+ concentration would further enhance Ca^{2+} influx by Na^+/Ca^{2+} exchange (DiPolo and Beaugé, 1983; Lagnado and McNaughton, 1990). However, judging by the observations of Sah et al (1989) a net outward current is induced only when a selective NMDA antagonist (but not a selective non-NMDA antagonist) is applied to CA1 pyramidal cells. The NMDA-receptors are probably the most important route for Ca^{2+} influx evoked by an ongoing glutamate release and our data certainly do not rule out the possibility of Ca^{2+} influx mediated by the non-NMDA receptor-coupled channels (Iino et al, 1990; Gilbertson et al, 1991; Burnashev et al. 1992; Burnashev et al, 1992). In any case, our results suggest that as a consequence of glutamate receptor-mediated channel activation, a rise in $[Ca^{2+}]_i$ triggers Ca^{2+}-dependent Ca^{2+} inactivation (Eckert and Chad, 1984), thus explaining the reduction in HVA Ca^{2+} currents.

4.6.3 Involvement of second messengers

In addition to the augmentation in $[Ca^{2+}]_i$ activated by ionotropic receptors, GM1 may activate the second messenger systems which also modulate $[Ca^{2+}]_i$.

Indeed, our results are in agreement with those reported by Lester and Jahr (1990). Using primary cell culture of CA1 pyramidal neurons, these investigators have reported that activation of G protein-coupled metabotropic glutamate receptors, can *directly* depress HVA Ca^{2+} currents. In brain slices (Nicoletti et al, 1986; Schoepp and Johnson, 1988; Palmer et al, 1988), as well as in primary culture of striatal neurones (Sladeczek et al, 1985), activation of quisqualate receptor-coupled to a G protein by glutamate has been shown to activate phospholipase C, resulting in IP_3 formation. An increase in IP_3 formation could release Ca^{2+} from intracellular Ca^{2+} storage pools.

However, Desai and Conn (1991) recently reported that activation of the metabotropic receptors by the glutamate analogue trans-1-amino-1,3-cyclopentanedicarboxylic acid (trans-ACPD) is insensitive to kynurenic acid. This discrepancy may reflect a highly specific inhibitory action of kynurenic acid on glutamate rather than trans-ACPD. Alternatively, it is also conceivable that some of the effects mediated by metabotropic receptors were mimicked by GM1 since GM1 stimulates phosphoinositide degradation, resulting in IP_3 formation (Leray et al, 1988).

4.6.4 Effects of GM1 on chloride conductances

In Xenopus oocytes, Sugiyama et al (1987 and 1989) have demonstrated that

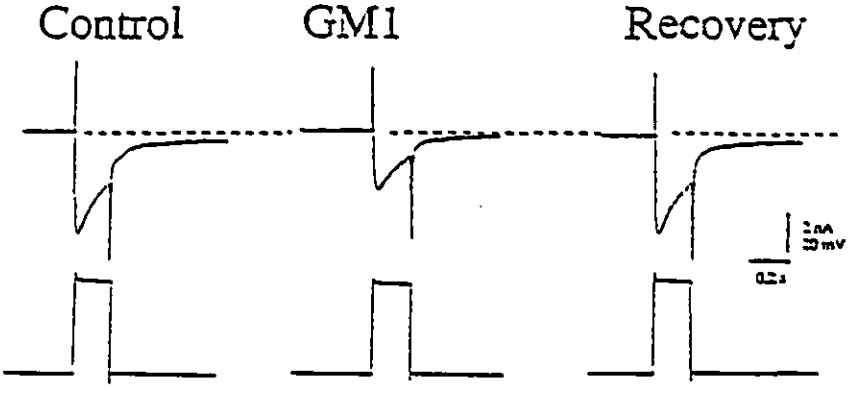
augmentation in $[Ca^{2+}]_i$, as a consequence of G protein-coupled glutamate receptor activation, stimulates Ca^{2+}-dependent Cl^- conductance. This type of Cl^- conductance was also observed in rat dorsal root ganglion neurons (Mayer, 1985), and in cultured mouse spinal cord and hippocampal pyramidal neurons (Owen et al, 1984, 1986, and 1988). Based on the results obtained from chloride and non-chloride loaded cells in our experiments, it is possible that such a Ca^{2+}-dependent Cl^- conductance is activated. However, the small difference in slope conductance observed between Cl^-- and non-Cl^--loaded cells (Fig. 4.7) indicate a minor involvement of Cl^-. However, blockade of GM1-induced reduction of HVA Ca^{2+} currents by bicuculline suggest a possible interaction between GM1 and GABA receptors, in particular $GABA_A$. At present, we have insufficient information to fully explain this observation, in view of the fact that GM1 does not alter spontaneous GABA release (Miu and Krnjević, 1991*a*).

In conclusion, our findings suggest that GM1 modulates HVA Ca^{2+} currents via activation of glutamate receptor-mediated channels, and perhaps in consequence of increased $[Ca^{2+}]_i$ results in the blockade of HVA Ca^{2+} currents.

4.7 Figures

Figure 4.1. GM1 attenuates HVA inward Ca^{2+} currents, recorded in CA1 neurons with CsCl-filled electrode. *A*) HVA inward Ca^{2+} currents (above) were elicited by constant depolarizing voltage commands (below). Ca^{2+} current was isolated by blocking Na^{+} currents with TTX (1 μM), and K^{+} currents with TEA (10 mM), CsCl (4 mM), and 4-AP (100 μM). *B* shows (for the same cell) the current-voltage (I/V) relation of the peak inward current (before, during, and after bath application of GM1, 1 μM) . Note that, GM1 reversibly reduced the net Ca^{2+} current over a wide voltage range, without any change in the voltage-dependence and the threshold for activation. Substantial recovery was seen after 30 min of wash. This cell was clamped at V_H -40 mV, as indicated.

A


Control
GM1
Recovery
V_H -40 mV

B

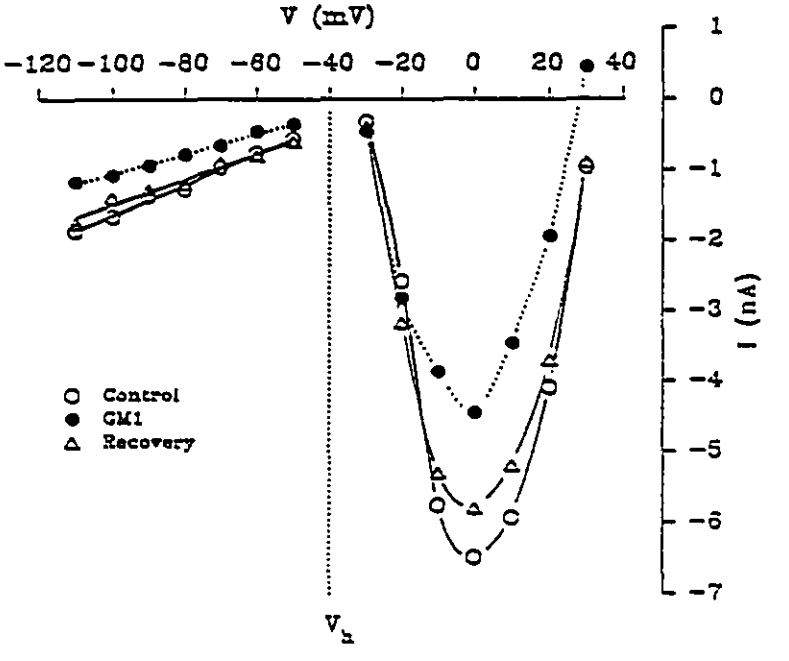
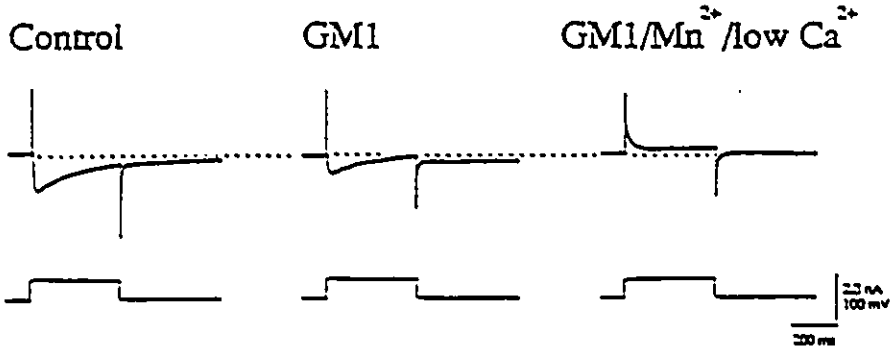

V (mV)
−120 −100 −80 −60 −40 −20 0 20 40
1
0
−1
−2
−3
−4
−5
−6
−7
I (nA)
Control
GM1
Recovery
V_h

Figure 4.2. *A*, HVA Ca^{2+} current is reduced by GM1 and abolished by Mn^{2+} and low Ca^{2+} perfusion. *B* from a different cell current-voltage relation of the peak inward HVA Ca^{2+} current during superfusion with control ACSF (open circles), GM1 (1 μM, filled circles), and Mn^{2+} (2.3 mM) and low Ca^{2+} (0.4 mM) solution (filled triangles). GM1 reduced the peak inward current over a wide voltage range with no change in the threshold for activation. There was a further reduction of the peak inward current in the presence of Mn^{2+} and low Ca^{2+}. Both cells were recorded with CsCl-filled electrode, and clamped at V_H -40 mV.

A


Control
GM1
GM1/Mn2+/low Ca2+

$V_H = -40$ mV

B

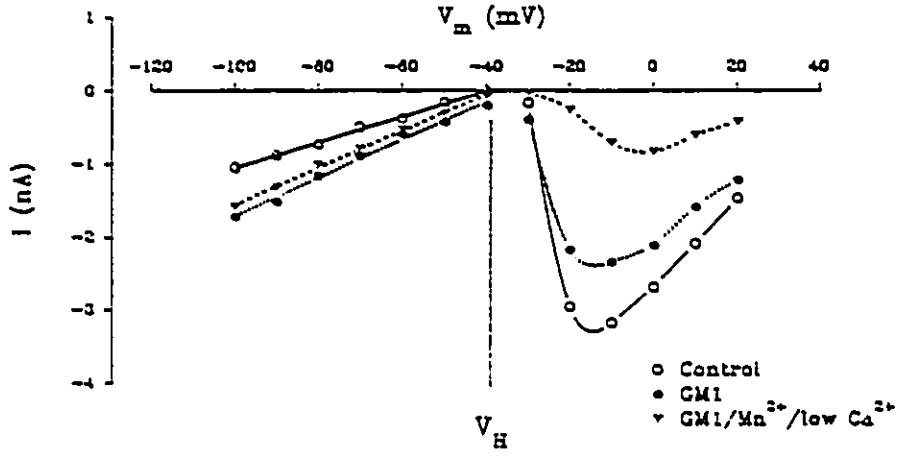

Vm (mV)
-120
-100
-80
-60
-40
-20
0
20
40
I (nA)
VH
Control
GM1
GM1/Mn2+/low Ca2+

Figure 4.3. Low Ca^{2+} and kynurenic acid block GM1-induced inward shift in I_H. *A*, recorded in the presence of TTX, shows inward shift in holding current (I_H) during GM1 (1 μM) perfusion, which was reversed by perfusate containing Mn^{2+} (2.3 mM) and low Ca^{2+} (0.4 mM; note also reversible block of the GM1-induced 'spontaneous' inward currents). *B*, recorded from another CA1 cell, shows the lack of GM1 effect on I_H in the presence of kynurenic acid (1 mM) applied approximately 10 minutes prior to GM1 perfusion. Also evident in this current trace are kynurenate-insensitive spontaneous inward currents. Both cells were impaled with CsCl-filled electrodes and clamped at -40 mV.

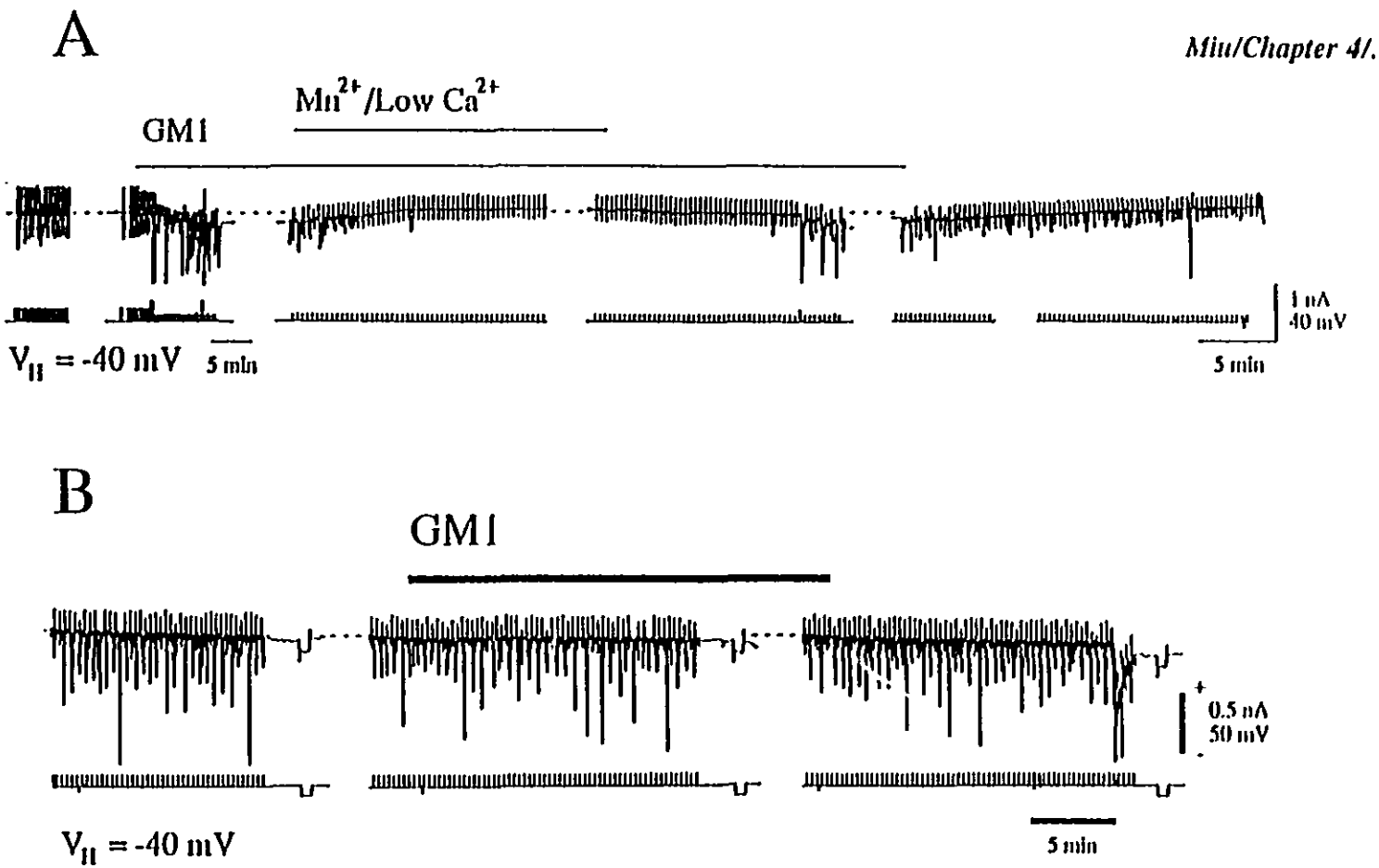
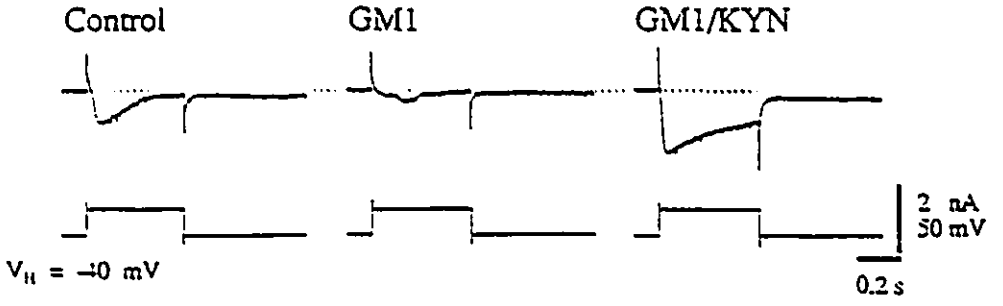

A
GM1
Mn^{2+}/Low Ca^{2+}
1 nA
40 mV
V_H = -40 mV
5 min
5 min
B
GM1
0.5 nA
50 mV
V_H = -40 mV
5 min

Figure 4.4. Kynurenic acid (1 mM; KYN) antagonizes GM1-induced attenuation of HVA Ca^{2+} currents. (*A*) Single traces of HVA Ca^{2+} currents (above) evoked by constant depolarizing pulses (below) during control, GM1, and GM1+KYN perfusion. Current-voltage plots (*B*) show net peak inward currents, its reduction by GM1 (1 μM, filled circles), and restoration by KYN (filled triangles) over a wide range of potentials. Also indicated in *B* is the outward shift in I_H and slight reduction in leak conductance (G_L) (in the negative range). This cell was recorded with CsCl-filled electrode, and clamped at V_H -40 mV as indicated.

A


Control
GM1
GM1/KYN
2 nA
50 mV
V_H = -40 mV
0.2 s

B

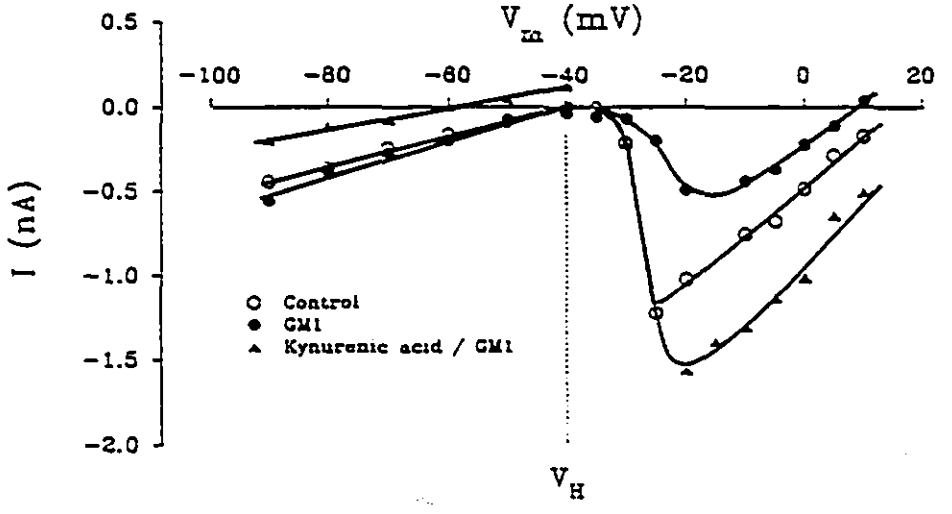

V_m (mV)
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-100
-80
-60
-40
-20
0
20
I (nA)
Control
GM1
Kynurenic acid / GM1
V_H

Figure 4.5. GM1-induced attenuation of HVA Ca^{2+} currents is more pronounced for the peak than for the 'steady state' current. Changes in the peak (*A*) and 'steady state' (*B*) inward currents (expressed as a percentage of control values) were obtained from 32 cells recorded with CsCl-filled electrodes. Bath perfusion with kynurenic acid (1 mM; KYN) alone had no significant effect on the peak and 'steady state' inward currents; whereas bicuculline (10 μM; BMI) alone significantly enhanced the peak inward current. However, both receptor antagonists prevented GM1 from reducing the peak inward current. * indicates statistical significance (see Results for the *P* values).

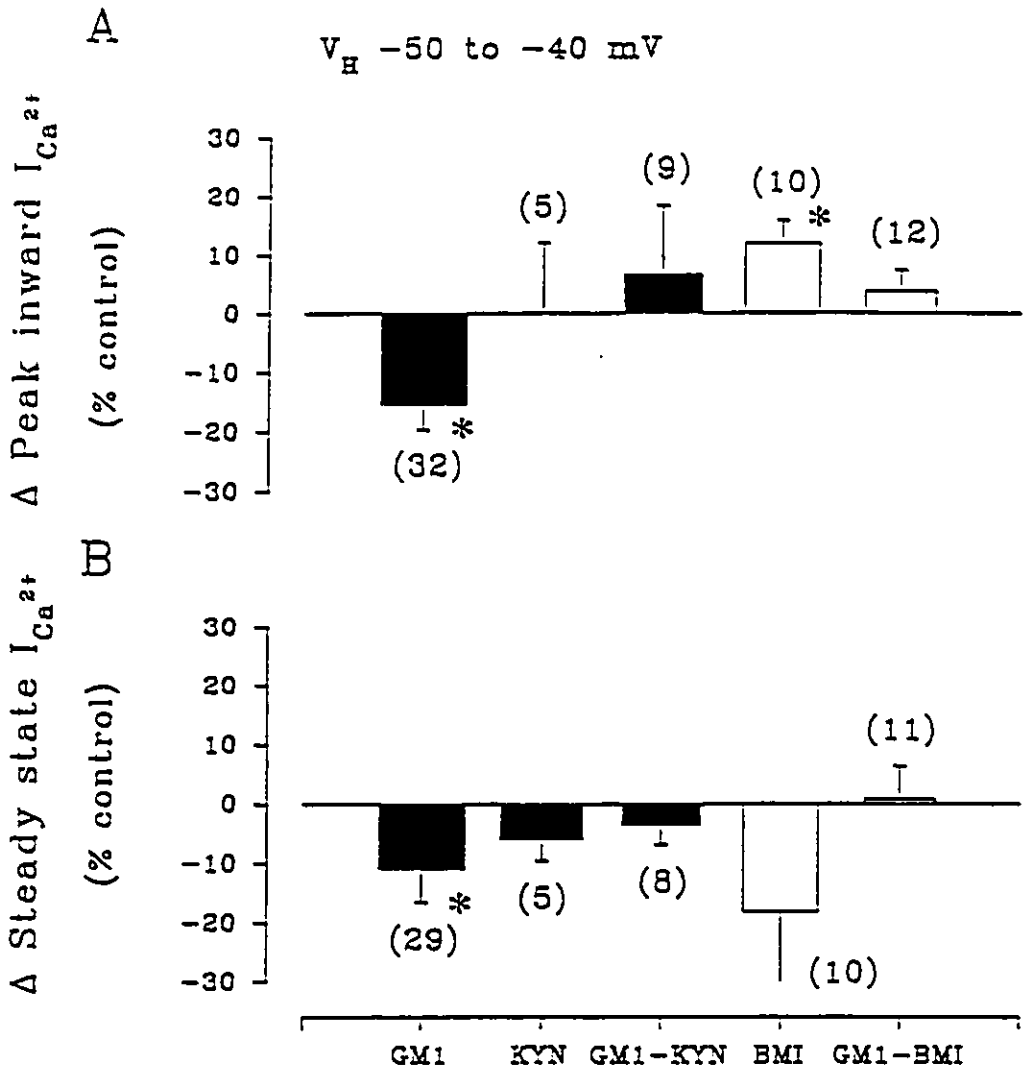

A
V_H −50 to −40 mV
Δ Peak inward $I_{Ca^{2+}}$
(% control)
30
20
10
0
−10
−20
−30
(32) *
(5)
(9)
(10) *
(12)
B
Δ Steady state $I_{Ca^{2+}}$
(% control)
30
20
10
0
−10
−20
−30
(29) *
(5)
(8)
(10)
(11)
GM1
KYN
GM1-KYN
BMI
GM1-BMI

Figure 4.6. Histograms summarizing GM1-induced inward shifts in holding current (ΔI_H) and increases in leak conductance (ΔG_L), and the effects of kynurenic acid (KYN) and bicuculline (BMI). The values represent the change in I_H and G_L from the control values. A total of 37 CA1 cells were recorded using CsCl-filled electrodes, and the holding potentials (V_H) ranged from -65 to -35 mV. Perfusion with kynurenic acid (1 mM; KYN) alone induced a net outward shift in I_H; whereas a net inward shift in I_H was observed with BMI alone (*A*). The GM1-induced inward shift in I_H was blocked by KYN but not by BMI. In *B*, neither KYN nor BMI alone had any significant effect on G_L. However, both antagonists blocked the GM1-induced increase in G_L. * indicates statistical significance (see Results for the *P* values).

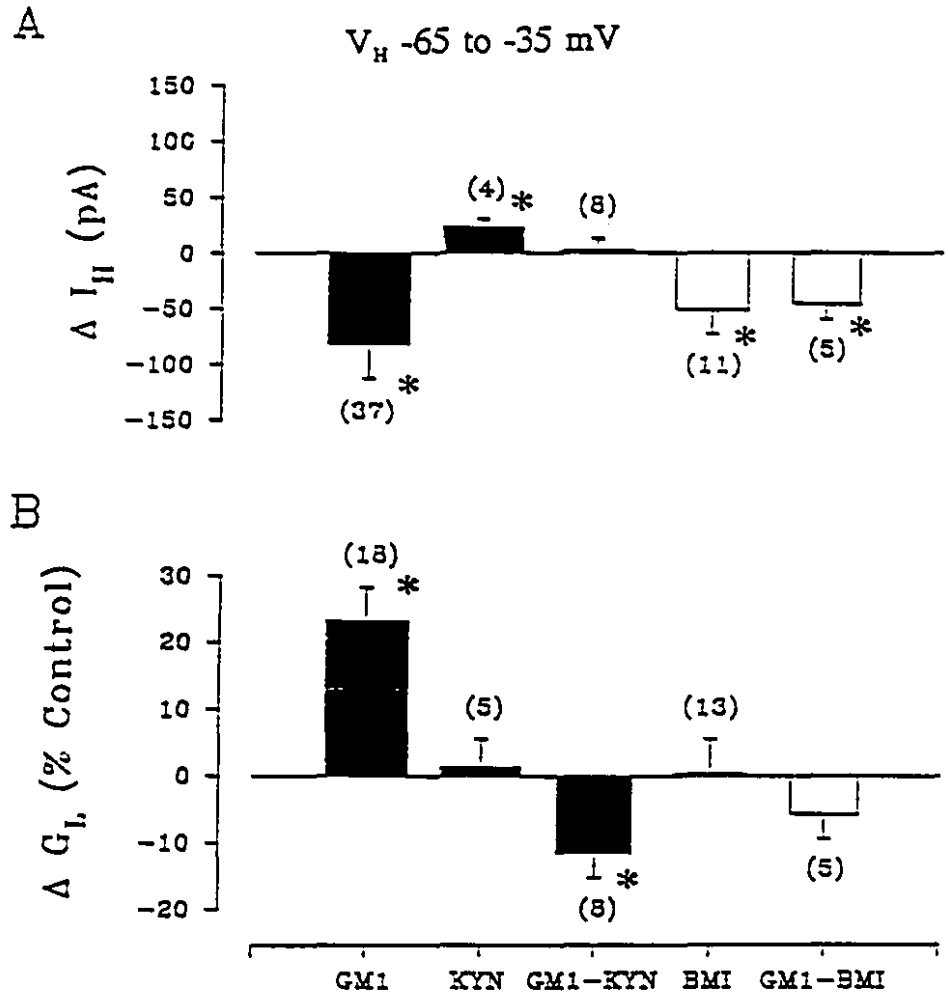
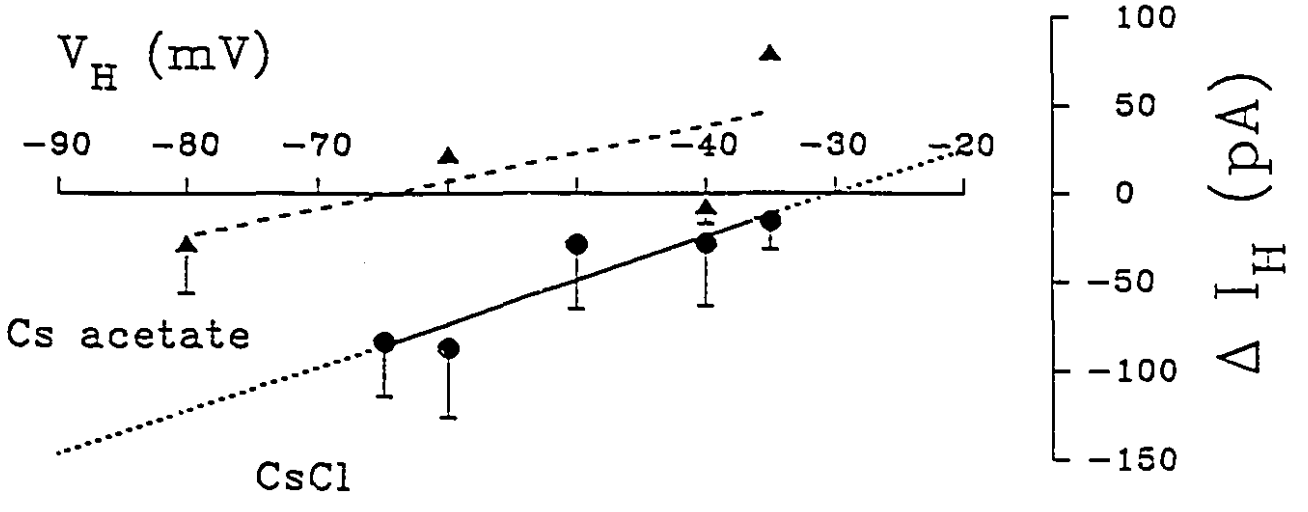

A
V_H -65 to -35 mV
Δ I_H (pA)
150
100
50
0
−50
−100
−150
(4) *
(8)
(37) *
(11) *
(5) *
B
Δ G_L (% Control)
30
20
10
0
−10
−20
(18) *
(5)
(13)
(8) *
(5)
GM1
KYN
GM1−KYN
BMI
GM1−BMI

Figure 4.7. Reversal potential for GM1-induced shift in holding current was more negative in non-chloride loaded cells than for the chloride loaded cells. Each point represents the mean ($\pm$ s.e.m.) change in I_H produced by GM1 at a given V_H, circles for CsCl-electrodes (total of 33 values). Examples for Cs acetate-electrodes (total of 9 values). From the interception of regression line fitted to those points, the reversal potentials were estimated at -31 mV for the Cl^- loaded cells and -63 mV for the non-Cl^- loaded cells.


V_H (mV)
−90
−80
−70
−40
−30
−20
Cs acetate
CsCl
100
50
0
−50
−100
−150
ΔI_H (pA)

4.8 References

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CHAPTER 5

5.1 GENERAL DISCUSSION

Monosialoganglioside (GM1) has been demonstrated to enhance the induction of long term potentiation (LTP) in rat hippocampal slices (Wieraszko and Seifert, 1985; Ramirez et al, 1990; Hwang et al, 1991); however, the mechanisms underlying LTP are still unclear. Hence this project was designed to assess the role of GM1 in synaptic transmission. Exogenous GM1 was dissolved in artificial cerebrospinal fluid (ACSF), and applied to rat hippocampal slices. Results obtained from synaptic transmission studies showed that GM1 selectively enhances the excitatory inputs while simultaneously reducing the inhibitory inputs (Chapter 3). The mechanism underlying the potentiation of excitatory inputs is probably due to an enhancement in glutamate release, as indicated by the increase in the frequency and amplitudes of the spontaneous miniature excitatory postsynaptic currents.

The enhanced glutamate release in the presence of GM1 is further demonstrated, in the voltage clamp studies, by the induction of a kynurenate-sensitive inward shift in the holding current (Chapter 4). Also shown in this study was a GM1-induced reduction in HVA Ca^{2+} currents. Hence, these observations suggest that activation of glutamate receptors (i.e., non-NMDA-, NMDA-, and metabotropic-receptors) augments intracellular Ca^{2+} concentration. Such an enhancement in the intracellular Ca^{2+} concentration would partially explain the reduction seen in HVA Ca^{2+} currents in the presence of GM1 (Eckert and Chad, 1984).

5.2 Exogenous Versus Endogenous GM1

The foremost question concerning the use of exogenous GM1 is whether or not these compounds can be incorporated into the plasma membrane and subsequently execute the same functions as the endogenous GM1. Fishman and colleagues in the late seventies examined this issue, using transformed mouse fibroblasts NCTC 2071 (Fishman et al, 1976; Moss et al, 1976). In their studies, NCTC 2071 cells were grown in chemically defined medium, which was specifically designed to prevent the synthesis of gangliosides. Consequently these cells did not respond to cholera toxin, as indicated by the absence of cyclic adenosine3',5'-monophosphate (cAMP) production. However, when incubated with exogenous GM1, these gangliosides were not only incorporated into the plasma membrane in a time- and concentration-dependent manner, but also bound cholera toxin, which subsequently activated the production of cAMP via adenylate cyclase. The ability of exogenous GM1 to be incorporated into the plasma membrane was later demonstrated in crude neuronal membranes (Toffano et al, 1980) and mitochondrial fraction of rat brain (Leon et al, 1981). Hence, most of the GM1-induced effects that we have observed are most likely mediated by the incorporation of exogenous GM1 into the plasma membrane. This action subsequently enables the newly incorporated GM1 to exert its actions by either i) direct interaction with membrane bound enzymes such as adenylate cyclase, or ii) degradation to its end products, which can be re-cycled for *de novo* synthesis of GM1 or function as inhibitors of second messengers.

5.2.1 Interaction with adenylate cyclase

Gangliosides have been shown by Partington and Daly (1979) to activate adenylate cyclase. In their studies, addition of mixed brain gangliosides to membrane preparations from rat cerebral cortex potentiated the basal adenylate cyclase activity by 50-95%, resulting in a substantial elevation of intracellular cAMP concentration. Since the concentration of GM1 required for half maximal activation of adenylate cyclase was approximately 50 μM, it is unlikely that 1 μM GM1 would be sufficient to potentiate adenylate cyclase activity significantly in our preparation. Moreover, the lack of GM1 effect on monosynaptic IPSP amplitudes further argues against any GM1-induced synthesis of cAMP because intracellular accumulation of cAMP has been shown to induce $GABA_A$ receptor desensitisation (Harrison and Lambert, 1989; Tehrani et al, 1989; Porter et al, 1990). The cAMP-induced desensitisation of $GABA_A$ receptor was further demonstrated by using cloned $GABA_A$ receptor subunits (Moss et al, 1992).

Moreover, in our studies, activation of calmodulin-dependent kinase II by GM1 would induce phosphorylation of cAMP-dependent phosphodiesterases (Davis and Daly, 1980; Yates et al, 1989; Higashi et al, 1992; Higashi and Yamagata, 1992), thereby facilitating the degradation of cAMP. Hence, this effect would further make the involvement of cAMP-dependent kinases in the GM1 mediated actions highly improbable.

5.2.2 GM1 degradation

More recently, it has been shown that newly incorporated GM1 can be metabolised

into its catabolic end products such as ceramide and sphingosine in cultured cerebellar granule cells (Riboni et al, 1992). The formation of sphingosine is of great interest for our studies since it blocks protein kinase C (PKC; Hannun et al, 1986; Kreutter et al, 1987; Hannun and Bell, 1989), as well as Ca^{2+}/calmodulin-dependent kinase II (Jefferson and Schulman, 1988), by acting on their regulatory subunits. Indeed, our previous data (Agopyan et al, 1992) have shown that GM1, acting via either its intact sphingosine moiety or its metabolic end-product sphingosine, blocks the phorbol ester-mediated PKC augmentation of HVA Ca^{2+} currents.

The fact that GM1 enhances excitatory synaptic transmission while simultaneously blocking the PKC-mediated actions seems paradoxical because activation of PKC and Ca^{2+}/calmodulin-dependent kinase II are involved in the Ca^{2+}-dependent transmitter release process, and consequently in the induction of LTP (Llinás et al, 1985; Publicover, 1985; Zurgil and Zisapel, 1985; Malenka et al, 1986 & 1987; Nichols et al, 1987; Shapira et al, 1987). If GM1, through its sphingosine moiety, blocks PKC and Ca^{2+}/calmodulin-dependent kinase II, then instead of an enhancement of the excitatory synaptic transmission, we should have observed a reduction (or even a blockade) of excitatory synaptic transmission. Moreover, an increase in the Ca^{2+} influx is known to be the major step in enhancing synaptic transmission (Harvey and MacIntosh, 1940; Katz and Miledi, 1967*a*, 1967*b*, and 1967*c*; Augustine et al, 1987). Hence, a GM1-induced reduction in HVA Ca^{2+} currents would not explain the facilitation of excitatory synaptic transmission by GM1.

On the basis of these arguments, one would expect that any agent which blocks the activity of PKC, Ca^{2+}/calmodulin-dependent kinase II, and HVA Ca^{2+} channels would reduce the efficacy of synaptic transmission. However, our data showed that GM1 facilitates excitatory synaptic transmission, which is mainly mediated by an increase in excitatory transmitter release. Since the GM1-induced augmentation in transmitter release was also observed in the presence of TTX, mechanisms other than the voltage-dependent Ca^{2+} influx via presynaptic HVA Ca^{2+} channels would be involved. These mechanisms most probably include phosphoinositide turnover, which is stimulated either directly by GM1 or indirectly by metabotropic glutamate receptors.

5.3 Mechanisms Underlying the Effects of Exogenous GM1

5.3.1 Phosphoinositide metabolism

Stimulation of phosphoinositide turnover by exogenous GM1 has been demonstrated in cultured chick neurons (Ferret et al, 1987; Leray et al, 1988). In these cells, incubation with mixed exogenous gangliosides (10^{-8} and 10^{-5} M) facilitated the production of inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is known to enhance intracellular Ca^{2+} release from cytosolic Ca^{2+} pools (Berridge and Irvine, 1984), while DAG activates PKC (Nishizuka, 1984). Consequently, GM1 should activate PKC and increase intracellular Ca^{2+}. Increased $[Ca^{2+}]_i$ would reinforce the activation of phospholipase C (Eberhard and Holz, 1988), and activate calmodulin (Trimble et al, 1991).

5.3.1.i Protein kinase C

The role of PKC in the modulation of synaptic transmission, however, is different from that of GM1 because PKC enhances both excitatory and inhibitory inputs (Malenka et al, 1986 & 1987; Corradetti et al, 1991; Agopyan and Agopyan, 1991; Agopyan et al, 1992). However, observations such as a) inhibition of PKC-mediated actions by GM1's sphingosine moiety (discussed above), and b) activation of Ca^{2+}/calmodulin-dependent kinase II by GM1 (Fukunaga et al, 1990; Higashi et al, 1992; Higashi and Yamagata, 1992), which subsequently exerts a negative feedback on PKC (Albert et al, 1984), suggest that overall, PKC activity is blocked by GM1. Therefore we believe that the principal mechanism underlying GM1-induced transmitter release is mediated by Ca^{2+}/calmodulin-dependent kinase II.

5.3.1.ii Ca^{2+}/calmodulin-dependent kinase II

As mentioned above, GM1 stimulates Ca^{2+}/calmodulin-dependent kinase II (CaMII). However, this effect is highly dose-dependent since low- ($\approx$ 25 μM) and high-doses (125-250 μM) stimulate and inhibit CaMII respectively (Fukunaga et al, 1990). More recently, Higashi and Yamagata (1992) proposed that the bimodal function of gangliosides is caused by a direct binding of gangliosides to the Ca^{2+}/calmodulin binding site of CaMII. At low ganglioside concentration, the Ca^{2+}/calmodulin binding site of CaMII is occupied by gangliosides thereby activating CaMII. However, as the ganglioside concentration increases, the Ca^{2+}/calmodulin binding site saturates thereby inactivating

CaMII.

In our studies, the GM1 concentration (usually 1 μM) applied to hippocampal slices was low; moreover, the actual concentration reaching the synaptic cleft may be much lower than 1 μM if one considers factors such as non-specific binding of exogenous GM1 in the slice. In view of these factors, Ca^{2+}/calmodulin-dependent kinase II is most probably stimulated rather than inhibited by GM1. Activation of Ca^{2+}/calmodulin-dependent kinase II could subsequently phosphorylate both synapsins and MAP-2, resulting in facilitation of transmitter release (see Introduction).

5.3.2 Indirect activation of glutamate receptors by GM1

In addition to the build-up of intracellular Ca^{2+} by a direct stimulation of phosphoinositide turnover, GM1 may also indirectly raise $[Ca^{2+}]_i$ through the activation of both ionotropic and metabotropic glutamate receptors (mGluR). This is indicated by our data which showed that the frequency of spontaneous miniature EPSCs, recorded from CA3 pyramidal cells, is consistently increased in the presence of GM1. The enhanced glutamate concentration in the synaptic clefts would therefore activate both types of glutamate receptors.

Activation of mGluRs coupled to G-proteins, in particular G_p, has been shown to enhance the breakdown of PLC-dependent phosphatydlinositol-4,5-bisphosphate (PIP_2), thereby generating metabolic end products such as IP_3 and DAG (Sladeczek et al, 1985; Nicoletti et al, 1986; Schoepp and Johnson, 1988; Palmer et al, 1988). This acts as a

positive reinforcement for PIP_2 turnover induced by GM1. Moreover, activation of G protein-coupled metabotropic glutamate receptors has been shown to directly depress HVA Ca^{2+} currents (Lester and Jahr, 1990).

Suppression of HVA Ca^{2+} currents via G protein activation is also consistent with our data which showed that GM1 reduces the HVA Ca^{2+} currents (Chapter 4). The GM1-induced reduction of the HVA Ca^{2+} currents, however, can be reversed in the presence of kynurenic acid which has been shown to block only the ionotropic glutamate receptors (Robinson et al, 1984; Bertolino et al, 1989). Nevertheless, more recent findings of multiple subtypes of metabotropic glutamate receptors in the mammalian brain, and the lack of pharmacological data on these subtypes, suggest a possible interaction between GM1 and one of the subtypes, which may be sensitive to kynurenic acid (Vecil et al, 1992).

5.4 Consequences of GM1 Application

When exogenous GM1 is applied to the hippocampal slices, it is incorporated into the plasma membrane of nerve terminals thereby stimulating phosphoinositide turnover, presumably through activation of phospholipase C. Formation of IP_3 would increase $[Ca^{2+}]_i$, which triggers a cascade of second messengers. Subsequent activation of these second messengers results in an augmentation of excitatory transmitter release, thereby potentiating the efficacy of excitatory synaptic transmission. The GM1-induced

augmentation in glutamate release would further contribute to the formation of IP_3 via activation of pre-synaptic metabotropic glutamate receptors, thus further enhancing transmitter release.

5.5 Effects of GM1 on Inhibitory Nerve Terminals

In contrast to the GM1-induced excitatory transmitter release, the spontaneous miniature IPSC studies showed that GM1 has no significant effect in altering both the amplitude and frequency of miniature IPSCs. This is further supported by the lack of GM1 effect on the evoked monosynaptic IPSP amplitudes (Chapter 3). In keeping with our observations, Frieder and Rapport (1981 & 1987) also demonstrated differential actions of GM1 on GABAergic, noradrenergic, and serotonergic nerve terminals. In their studies, anti-GM1 antibodies greatly enhanced the depolarization-induced GABA release from rat brain slices; however, the depolarization-induced release of norepinephrine and serotonin was not significantly altered by anti-GM1 antibodies (Frieder and Rapport, 1981).

These opposite actions of GM1 on the GABAergic, noradrenergic, and serotonergic nerve terminals (and in our studies, the glutamatergic nerve terminals) could be explained by the differences in the distribution of a) GM1 in these specialized nerve terminals, or b) GM1 with respect to ion channels in the plasma membranes, as suggested by Frieder and Rapport (1981). Alternatively, another explanation for these differences would be that not all of the second messengers (discussed in this Thesis) activated by GM1 are

present in the inhibitory synapses.

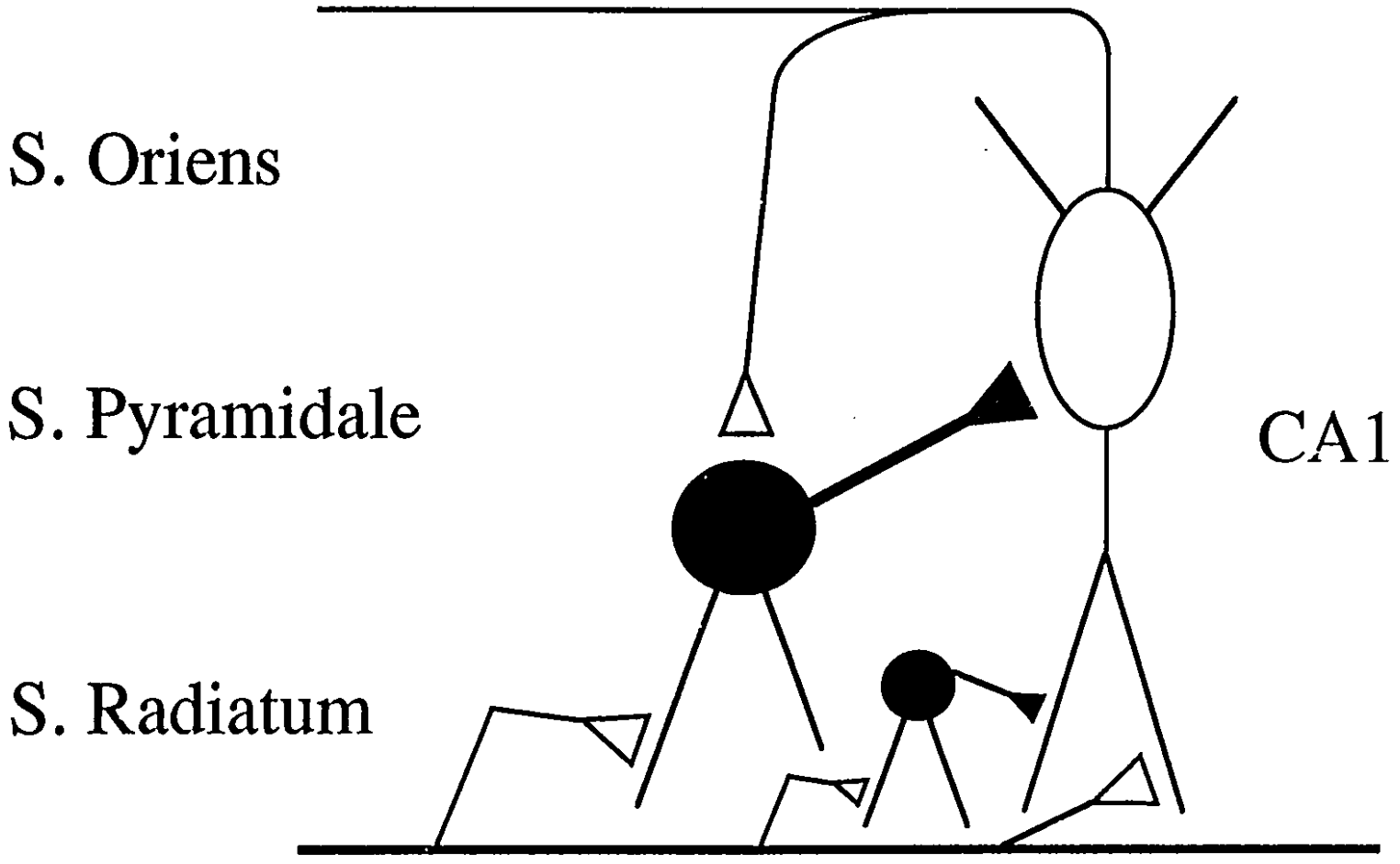
Therefore one can interpret on the basis of these findings that under normal physiological conditions, GM1 modulates depolarization-induced GABA release, perhaps, by acting in a similar manner as the presynaptic $GABA_B$ receptors (Peet and McLennan, 1986). Hence, agents such as the anti-GM1 antibody could conceivably interrupt this negative modulation by GM1, resulting in the facilitation of GABA release.

5.6 Conclusions

Thus far, I have discussed GM1 actions with respect to the excitatory and inhibitory inputs as separate entities. The experimental evidence that I have presented so far suggest that GM1 improves the efficacy of synaptic transmission. This phenomenon is perhaps not too surprising in view of the fact that changes in the plasma membrane concentration of GM1 at the nerve terminals of mammalian nerve cells via exposure to either catalytic enzyme such as neuraminidase or exogenous GM1 ultimately reduces the efficacy of synaptic transmission or enhances mnenomic processes such as long term potentiation respectively (Wieraszko and Seifert, 1984, 1985, & 1986; Ramirez et al, 1990; Hwang et al, 1991). The GM1-induced augmentation of excitatory inputs may subsequently influence the inhibitory inputs.

Due to the anatomical location of the inhibitory interneurons, as depicted by the diagram of the hippocampal circuitry in figure 5.1, GM1-induced facilitation of the

Figure 5.1. A simplified diagram of the hippocampal circuitry. The black circles represent inhibitory interneurons. Not shown in this diagram are the basal inhibitory interneurons and excitatory collateral inputs from adjacent CA1 neurons.


S. Oriens
S. Pyramidale
S. Radiatum
CA1

excitatory inputs will affect these cells via the feed-forward (Alger and Nicoll, 1982) and recurrent pathways (Kandel et al, 1961; Andersen et al, 1964*a* & 1964*b*). It is therefore conceivable that in spite of an increased barrage of excitatory inputs onto the inhibitory interneurons induced by GM1, their firing (or more importantly the transmitter releasing mechanism) is sufficiently depressed to reduce further GABA release, perhaps by activation of presynaptic $GABA_B$ receptors (Peet and McLennan, 1986). This speculative action of GM1 may explain the improved efficacy of synaptic transmission, thereby enhancing learning and memory.

5.7 References

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CHAPTER 6

6.1 SUMMARY AND ORIGINAL CONTRIBUTIONS*

Monosialogangliosides (GM1) are very abundant in the brain, particularly in the plasma membrane of presynaptic nerve terminals. However, the functional importance of GM1 has not been studied extensively. Most of the studies reported in the literature have concentrated mainly on the biochemical effects of GM1, following the discovery that catabolic enzyme deficiency results in excessive accumulation of various gangliosides.

The studies reported in this thesis are the first detailed account of the functional role of GM1 from a physiological perspective. As such, they have shown that:

1. GM1 selectively facilitates the excitatory synaptic inputs (EPSPs)*, while simultaneously reducing the inhibitory inputs (IPSPs)* to CA1 pyramidal neurons of hippocampal slices (Chapter 3). Although numerous investigators have reported the importance of GM1 in maintaining synaptic transmission, as well as facilitating the induction of long term potentiation, the exact mechanisms underlying these processes were not elucidated. Most of the hypotheses concerning the above mentioned actions of GM1 in synaptic transmission processes were, at best, based on assumptions derived from various biochemical and biophysical studies.
2. The GM1-induced facilitation of EPSPs is most probably caused by an increase in glutamate release* (Chapter 3). This is supported by the findings that GM1 enhanced the frequency and amplitudes of both

TTX-sensitive spontaneous EPSPs in the CA1 neurons* and TTX-insensitive spontaneous miniature EPSPs in CA3 neurons* (Chapter 3). These spontaneous postsynaptic responses were abolished by kynurenic acid*, a non-selective glutamate receptor antagonist, and therefore glutamate mediated (Chapter 3).

3. GM1-induced changes in transmitter release have been reported in previous studies using synaptosomal preparations, as well as brain slices; however, these investigators did not study glutamate release. Although we did not directly measure glutamate release, there was, nevertheless, sufficient evidence pointing to a GM1-induced enhancement of glutamate release since most of the enhanced responses were antagonised by kynurenic acid* (Chapter 3 & 4).

4. In the presence of TTX, GM1 transiently potentiated inward currents evoked in CA1 neurons by exogenous glutamate (iontophoretic applications)*, thereby suggesting another possible role for GM1 in modulating postsynaptic ligand-receptor interactions. A similar conclusion was initially reached by another group of investigators; however, subsequent studies by the same group led to a revised notion that GM1 does not alter receptor binding affinity for glutamate, but instead stimulates glutamate transport.

5. The reduction of IPSPs by GM1 was only observed when the excitatory inputs were active* (Chapter 3). In the presence of TTX, GM1 had no

effect on the electrically evoked (monosynaptic)* and the spontaneous miniature* inhibitory postsynaptic responses. Although GM1 has been implicated in the induction of epileptiform activity, so far no one had elucidated the mechanism underlying this action of GM1. The present study, by showing that under relative physiological conditions, GM1 promotes a high level of excitatory inputs while suppressing the opposing inhibitory actions*, and provides a possible mechanism of epileptogenesis.

6. The selective reduction in the IPSPs by GM1* (Chapter 3) is consistent with other reports which showed that GABA release evoked by high K^+-induced depolarization is enhanced when exposed to anti-GM1 antibodies.

7. Since transmitter release is highly dependent on Ca^{2+} influx into the nerve terminals, we examined whether or not the GM1-induced augmentation in glutamate release could be due to a general enhancement of Ca^{2+} currents. Previous studies using cultured cells showed that GM1 enhances Ca^{2+} influx, measured by the radiolabelled $^{45}Ca^{2+}$ technique; however, due to technical limitations, the mechanism underlying this process could not be determined. Ca^{2+} influx is usually mediated via either ligand-gated receptors (e.g. glutamate) or HVA Ca^{2+} channels. In chapter 4, we show that HVA Ca^{2+} currents recorded by SEVC in CA1 pyramidal neurons were actually reduced by GM1*. Moreover, the GM1-induced reduction of HVA Ca^{2+} currents is usually coupled with an inward shift in the holding currents*, which indicates possible activation of glutamate-gated channels.

Indeed, kynurenic acid attenuated the effects of GM1 on HVA Ca^{2+} currents*, thereby suggesting that the enhanced Ca^{2+} influx through glutamate-gated channels induced a Ca^{2+}-dependent inactivation of HVA Ca^{2+} currents.

In summary, under physiological conditions, GM1 appears to modulate synaptic transmission presynaptically by enhancing glutamate release* and reducing inhibitory inputs*. This negative modulation of the inhibitory inputs appears to be mediated by changes in ongoing excitation of inhibitory interneurons*.