CHAPTER I

INTRODUCTION

1. Epilepsy

Epilepsy is a chronic condition whose major clinical manifestation is the occurrence of epileptic seizure characterized by sudden and usually unprovoked attacks of subjective experiential phenomena, altered consciousness, or involuntary movements (McNamara, 2001). Seizures result from paroxysmal and disordered discharges of cerebral neuron and are a common sign of brain dysfunction (Perucca, 2000). The behavioral manifestations of a seizure are determined by the function normally served by the cortical site at which the seizure arises. The causes of seizures are many and include the full range of neurological diseases, from infection to neoplasm and head injury. The term "epilepsy" is not usually applied to such patients unless the later develop chronic seizures (Griggs, 2001; Porter and Meldrum, 2001).

1.1 Incidence

Epilepsy affects about 50 millions worldwide (Doherty and Dingledine, 2002). The incidence is highest among young children and the elderly, and men are affected slightly more often than women (1.5: 1) (Griggs, 2001; Steinhoff et al., 2003). Based on epidemiological studies in the United States, about 30% of all people living to the age of so will be diagnosed with epilepsy (Kandel, Schwartz and Jessell, 2000).

1.2 Etiology

Epilepsy results from many conditions and mechanisms. It is not possible to identify a specific cause, although focal seizures imply a cerebral

injury or lesion. The most common specific lesions are hippocampal sclerosis, gangliomas and glial tumors, cavernous malformations, neuronal migrational defects (cortical dysplasia) and encephalitis, cerebral trauma and hemorrhage (Griggs, 2001). Not all patients with cerebral lesion becomes epileptogenic is poorly understood (Griggs, 2001).

1.3 Classification of Seizures and Epilepsies

Seizures have been classified in several ways: according to their supposed etiology and site of origin, on the basis of their clinical form, frequency, or eletrophysiologic correlates (Victor and Ropper, 1997). The most widely used classification scheme was first proposed by Gastaut in 1970 and was then refined repeatedly by The Commission on Classification and Terminology of the International League Against Epilepsy (Comission, 1981). This classification, based mainly on the clinical form of the seizure and its electroencephalogram (EEG), has been adopted worldwide and is generally referred to as the International Classification of Epileptic Seizure.

Seizures can be classified into large two categorized: Partial Seizure, those that originates in a small group of neurons limited to part of the cerebral hemisphere and Generalized Seizure, those that involve the cerebral cortex diffusely from the beginning (Dreifuss and Fountain, 1999; McNamara, 2000).

Numerous factors that affect the type and severity of seizures are ignored in the seizure classification (Kandel, Schwartz and Jessell, 2000). Such factors as the underlying etiology of the seizures, the age of onset, and family history all contribute to the clinical characteristics of epileptic syndromes (Commission, 1989). More than 40 distinct epileptic syndromes have been identified.

2. Mechanism of Epileptogenesis

Epileptic seizures result from excessive discharge in a population of hyperexcitable neurons. Most epileptic seizures are due to discharges generated in cortical and hippocampal structures (Avanzini and Franceschetti, 2003). The clinical expression of a seizure depends on its site of origin, time course, and discharge propagation (Avanzini and Franceschetti, 2003). The charges in neuronal excitability that underlie epileptogenesis not only induce abnormal activity in individual neurons but also recruit a critical mass of hyperexcitable cells in highly synchronized activities that are propagate through normal or pathological pathways. The mechanisms of epilepsy and normal brain function are interlinked (Najm, Ying and Janigro, 2001; Murashima, Yoshii and Suzuki, 2002).

2.1 Hyperexcitability

Two principal and complementary mechanisms determine neuronal hyperexcitability (Najm et al., 2001): (1) intrinsic membrane properties of neurons and ratio of inhibitory versus excitatory synapes; (2) extracellular levels of membrane permeant ions, and molecules available for neurotransmission play a role. Thus, the control of neuronal excitability depends on numerous factors, including gating properties and voltage-dependancy of ion channels, density of functional synapses concentrations of ions, and availability of mechanism of clearance of ions and neurotransmitters from the extracellular space (Najm et al., 2001; Avanzini and Franceschetti, 2003).

The observation that neurons in an epileptic neuronal aggregate consistently discharge in the form of protracted bursts of action potentials has been central to the investigation of epileptogenic cell mechanism (Medvedev, 2002). These bursts were called “paroxysmal depolarization shifts”, initially

described by Matsumoto and Ajmone-Marsan (1964). In focal epilepsies, paroxysmal depolarization shifts are a reliable marker of an established epileptogenic condition (Prince, 1985). Experimental epileptogenic procedures, such as the blockade of GABA-mediated inhibitory neurotransmission, or the potentiation of transmission by excitatory amino acids with selective agonists such as kainate, ibothenate, or NMDA can induce generalised phasic activity resembling paroxysmal depolarization shifts in cortical cells (Avanzini and Francesschetti, 2003). Similar effects are seen with epileptogenic agents that acts on the intrinsic mechanisms underlying membrane excitability, such as drug that increase sodium or calcium depolarizing currents or that reduce hyperpolarising potassium currents (Avanzini and Francesschetti, 2003).

The relevance of these mechanisms to human epilepsies is discussed in relation to the different types of voltage-gated and ligand-gated channels.

2.2 Sodium Channels

In the nervous system, voltage-gated ion channels control the flow of cations across surface and internal cell membranes (Barchi, 1998). Of these, the Na^+ channel is arguably of principal importance (Kwan, Sills, and Brodie, 2001). The neuronal Na^+ channel has a multi-subunit structure that forms a Na^+-selective, voltage-gated pore through the plasma membrane. The protein structure undergoes conformational alterations in response to changes in membrane potential, regulating conductance through the intrinsic pore (Ragsdale and Avoli, 1998).

The transient, fast-inactivating ionic current that flows through sodium channels, which causes the rising phase of the action potential, is associated with a slow-inactivating component that also has an important role in membrane excitability (Avanzini and Franceschetti, 2003).

A pathophysiological role of sodium channel in human epilepsies was originally suggested on the basis of indirect observations, such as the inhibitory effect of clinically effective antiepileptic drugs on sodium currents (Avanzini and Franceschetti, 2003). Phenytoin, the first generation antiepileptic drugs and newer agents have been found to inhibit sodium channels (Ragdale and Avoli, 1998), some of which seem to act preferentially on the persistent of the sodium current (Chao and Alzheimer, 1995; Taverna et al., 1999).

Studies of surgically resected human tissue have shown that changes in the ratio between different sodium channel subtypes are associated with drug-refractory seizures in temporal-lobe epilepsy (Lombardo et al, 1996). Moreover, in rat, changes in the kinetics of sodium-channel activation are associated with the development of epilepsy during kindling (Vreugdenhil, Faas and Wadman, 1999). The neuronal Na^+ channel represents one of the most important targets for antiepileptic drug action (Kwan et al., 2001).

2.3 Potassium Channels

Neuronal K^+ channels are large protein complex that form tetrametric structure, the monomers of which are structurally and genetically related to the α-and α1-subunits of the Na^+ and Ca^+ channel, respectively (Barchi, 1998). The association of four subunits(monomers) in the neuronal membrane is required for the formation of a K^+-sensitive pore and, therefore, channel function. More than 40 distinct K+ channel subunits have been identified, together with several auxillary subunits (Cosford et al., 2002). Given heterologous arrangement, it is possible that countless populations of K^+ channels, with individual functions and distributions, are expressed in the mammalian brain (Pongs, 1999). To date, 80 or more K+ channel related genes have been identified in the human genome, and genetic, molecular,

physiological and pharmacological evidence now exists to support a role for some of these K+ channels in the control of neuronal excitability and epileptogenesis (Wickenden, 2002).

At the neuronal level, K^+ channels are responsible for the action potential down stroke or, more specifically, repolarisation of the plasma membrane in the aftermath of Na^+ channel activation (Pongs, 1999).

Epileptiform discharges can be easily induced in tissue preparations *in vitro* by perfusion with K^+-channel blockers, such as extracellular tetraethylammonium or intracellular caesium ions, or by increasing the extracellular potassium concentration, thus reducing the strength of the outward potassium currents as a result of the decrease intracellular/extracellar concentration gradients (Avanzini and Franceschetti, 2003). Direct activation of voltage-dependent K^+ channels hyperpolarises the neuronal membrane and limit action potential firing (Porter and Rogawski, 1992). Accordingly, K^+ channels activators have anticonvulasant effects in some experimental seizure models (Gandolfo et al., 1989, Rostock et al., 1996), whereas K^+ channel blockers precipitate seizures (Yamaguchi and Rogawski, 1992).

KCNQ2 and KCNQ3 are voltage-gated K^+ channels expressed predominatly in the central nervous system (CNS). They can be found both pre- and post-synaptically in brain regions that are known to be important for the control of neuronal network oscillations and synchronization (Cooper et al., 2001). Several lines of evidence suggest that KCNQ2/Q3-based M-currents play an important role in the control of neuronal excitability and epileptogenesis. Mutation in the KCNQ2/KCNQ3 channels have been reported in benign neonatal familial convulsions, a generalized epilepsy syndrome (Rogawski, 2000).

Potentiation of voltage-gated K+ channel currents may prove to be an important target for future antiepileptic drug development (Wickenden, 2002).

2.4 Calcium Channels

Voltage-dependent Ca^{2+} channels (VDCCs) are critical for nerve function (Catterall, 2000). By coupling changes in the membrane potential to the influx of the pivotal "second messenger" Ca^{2+}, VDCCs represent the primary route for translating electrical signals into the biochemical events underlying key processes such as neurotransmitter release, cell excitability and gene expression (Jones, 2002).

High-threshold Ca^{2+} channels are subclassified by their pharmacological properties into L-, N-, P-, Q-, and R- types (Catterall, 1995). These channels are distributed throughout the central nervous system (CNS) on dendrites, cell bodies, and nerve terminals, The N-, P- and Q-type channels, in particular, have been implicated in the control of neurotransmitter release at the synapse (Stefani et al, 1997). The low-threshold T-type Ca^{+} channel is expressed predominantly in thalamocortical relay neurones, where it is believed to be instrumental in the generation of the rhythmic 3-Hz spike-and-wave discharge that is characteristic of generalized absence seizures (Coulter et al., 1989). Some experiments have shown that over expression of the low threshold calcium current in the thalamic-cell populations may be the cause of spike-wave discharge in the genetic absence rats (Avanzini et al., 1989).

Finally, calcium-dependent currents in intrinsically bursting neurons of the CA3 contributed these neurons to epileptic synchronization (Avanzini and Franceschetti, 2003)

2.5 GABA receptors

The two main types of ionotropic GABA receptors ($GABA_A$ and $GABA_B$) are coupled to chloride and potassium ionophores, respectively (Princivalle, 2000). A third receptor subtype, $GABA_C$, has also been reported but its physiological function in unclear at present. The inflow of chloride ions and out flow of potassium ions prompted by binding of GABA to its receptors leads to membrane hyperpolarization and inhibitory postsynaptic potentials (IPSPs). In general, activation of the GABAergic system causes neuronal inhibition and prevents epileptiform activity (Najm et al., 2001).

Considerable evidence suggests that impaired GABA function can cause seizures and may be implicated in some types of epilepsies. The molecular analysis of GABA receptors has shown that changes in the expression of $GABA_A$ receptor subunits occur in granule cells and in the molecular layer of rats during development of chronic epilepsy after kainic-acid-induced status epilepticus (Sperk et al., 1998). Studies on tissue resected from patients with mesial temporal lobe and neocortical epilepsies show reductions in the $GABA_A$ receptors (Mcdonald et al., 1991; Johnson et al., 1992).

Although supported by experimental data, the hypothesis that impaired GABA transmission leads to epileptic discharges is not unequivocally relevant to human epilepsies. In some instances, the organization of the underlying circuitry enables GABA neurotranssmission to be proepileptogenic rather than antiepileptogenic (Avanzini and Franceschetti, 2003).

Several antiepileptic drugs exert their effects, at least in part, by actions on the GABAergic system. Increased GABA synthesis, increased releases, allosteric receptor facilitation, and induced inactivation have all been implicated in the mechanisms of action of commonly used agents (Sills et al.,

1999). The GABA system also represents the most successful target for the rational design of novel antiepileptic compounds (Loscher, 1998).

2.6 Excitatory Amino Acid (EAA) Receptors

Glutamate and aspartate, the two major EAA neurotransmitters in the cerebral cortex, act through various receptor subtypes, the subunit composition of which determines their selective ionic permeability and the kinetics of the related ionic currents (Madden, 2002). Based on their pharmacologic and physiologic properties, the neuronal glutamate receptors are organised into two classes: ionotropic and metabotropic. The ionotropic receptors can be divided into two subpopulation: those that respond to α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) or kainic acid (KA) and those that respond to N-methyl-D-aspatate (NMDA) (Meldrum, 2000).

Because of the powerful influence of glutamatergic neurotransmission and the quasi-ubiquitous expression of glutamate receptors in the mammalian brain, it has been proposed that glutamatergic neurotransmission may play an important role in the pathogenesis of a variety of CNS disorder(Najm et al., 2001). Altered EAA neurotransmission, mediated primarily by glutamate, is a major cause of the imbalance of excitation and inhibition which characterizes both early development and epileptogenesis (Raol, Lynch and Brooks-Kayal, 2000).

The cellular mechanisms underlying cortical dysplasia (CD) epileptogenesis and, in particular, the role of AMPA/NMDA receptors in seizure expression, have been recently investigated in dysplasia cortex resected from patients suffering from medically intractable epilepsy. A correlation was found between cytoarchitectural abnormalities and specific NMDA-(NR1 and NR2A/B) and AMPA-(Glu2-3) receptor subunits (Ying et al., 1998). These

studies offer indirect evidence of a differential expression of some NMDA/AMPA-receptor subunits in the dysplastic neurons. The degree of NR1 and NR2A/B receptor in focal epileptogenesis was assayed. The densities of NR2A/B, but not NR1A subunits, are higher in resected cortical areas with EEG-proven epileptogenesis compared with neighboring nonepileptic cortex. NR2 subunit is co-localized with with NR1 protein, thus providing evidence of a potential functional substrate for a hyperexcitable NMDA receptor (Najm et al., 2000, Doi et al., 2001).

Experiments in various animal models have shown that sprouted mossy fibers make synaptic contacts in ectopic location, and thus from an excitatory feedback circuit (Franck et al., 1995). In general the information drawn from studies of mesial-temporal-lobe epilepsy supports the concept that epilepsy-related plasticity and changes in the molecular structure of receptors and channels are possible determinants of epileptogenic progression (Avanzini and Franceschatti, 2003). A straightforward cause-effect relation between sprouting and epileptogenesis is, however, challenged by the fact that experiment interventions that prevent sprouting do not impair the acquisition of epileptic properties (Avanzini and Franceschatti, 2003).

3. NMDA Receptors

Glutamate acts postsynaptically at several receptor types that are named for their prototypic pharmacological agonists (Dingeline et al., 1999). One major subtype is the N-methyl-D-aspartate (NMDA) receptor. This receptor is crucial to many forms of a process known as excitatotoxicity, during which the inability to respond properly to elevations in synaptic concentrations of glutamate overexcited neurons, leading to neuronal death (Lynch and Guttmann, 2002).

Glutamate is synthesized from glutamine by the action of the enzyme glutaminase in glutamatergic neurones (Daikin and Yudkoff, 2000). Following synaptic release, glutamate exerts its pharmacological effects on several receptors, classified into ionotropic and metabotropic families. Glutamate is removed from the synaptic cleft into nerve terminals and glial cells by the action of several specific transporters (Meldrum et al., 1999). Glial glutamate uptake is of principal importance. Glial cells convert glutamate into glutamine by the action of glutamine synthetase. Glutamine is subsequently transferred to glutamatergic neurons, completing the cycle (Daikin and Yudkoff, 2000).

Glutamate binds to both ion channel-associated (ionotropic) and G-protein-coupled (metabotropic) receptor types, which mediate fast excitatory and second messenger-evoked transmission, respectively (Cartmell and Schoepp, 2000). Ionotropic glutamate receptors are comprised of various combinations of subunits forming tetrameric and pentameric arrays (Kwan et al., 2001). They are classified into three specific subtypes, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA), which form ligand-gated ion channels, permeable to Na^+ and, depending on subtype and subunit composition, Ca^{2+} ion (Trist, 2000).

The AMPA and kainate subtypes of glutamate receptor are implicated to fast excitatory neurotransmission, where as the NMDA receptor, quiescent at resting membrane potential, is rescruited during periods of prolonged depolarization (Meldrum, 2000). The metabotropic family of glutamate receptors, also classified into three distinct subtypes (Group I, II, III), and G-protein linked and predominantly presynaptic, possibly controlling neurotransmitter release (Meldrum, 2000).

The NMDA receptor is an important target for drug development, with agents from many different classes acting on this receptor (Lynch and Guttmann, 2001). While the severe side effects associated with complete

NMDA receptor blockade have limited clinical usefulness of most antagonists, the understanding of the multiple forms of NMDA receptors provides an opportunity for development of subtype specific agents with potentially fewer side effects.

The N-methyl-D-Aspartate (NMDA) receptor is a heteromeric ligand-gated ion channel that interacts with multiple intracellular proteins by way of different subunits (Loftis and Janowsky, 2003). NMDA receptors (NMDARs) are concentrated at postsynaptic sites, although some appear to be presynaptic (Liu et al., 1994). Neurotransmission involving NMDA receptors has been implicated in a variety of unique roles: (1) NMDA receptors activation associated with long-lasting changes in synaptic strength (Ali and Salter, 2001), (2) organization of afferent fibers with respect to target neurons during development (Collingridge and Singer, 1990), and (3) participation in glutamate neurotoxicity (Marino and Conn, 2002).

3.1 Molecular Diversity

The number of subunits in each heteromeric glutamate receptor channel is still unknown (Yamakura and Shimoji, 1999). However, the glutamate receptor channels are proposed to be composed of four or five subunits (Ferrer-Montiel and Montal, 1996, Stephenson, 2001).

NMDA, AMPA, and kainate receptor subunits are encoded by at least six gene families as defined by sequence homology: a single family for AMPA receptors, two for kainate, and three for NMDA (Dingledine et al., 1999; Stephenson, 2001).

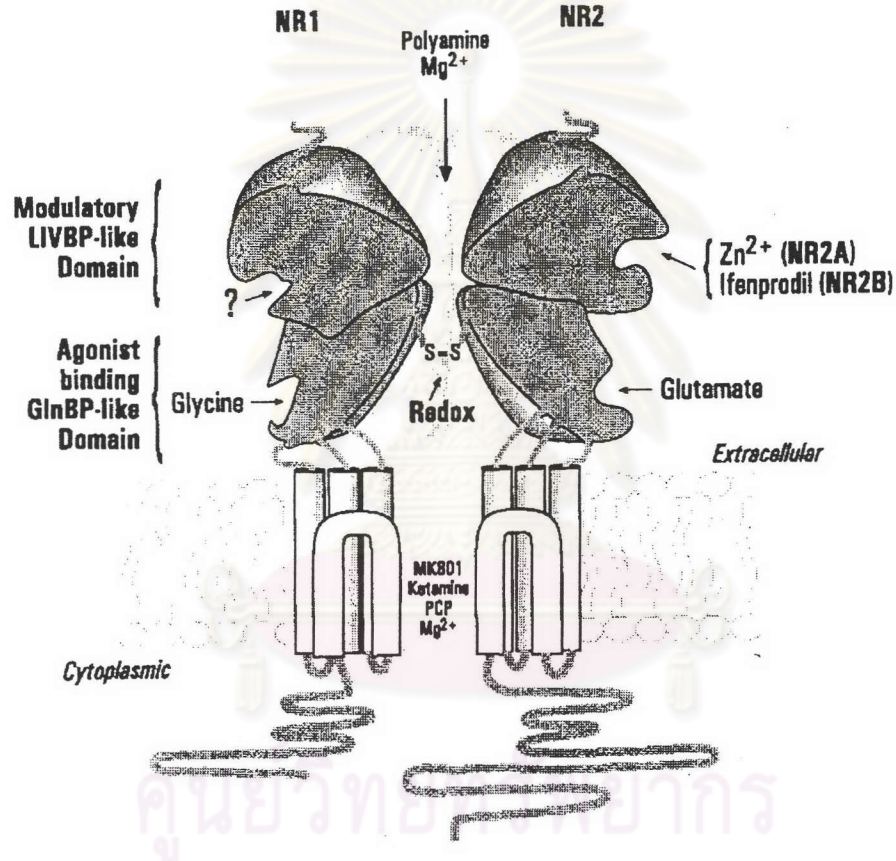


Figure 1.1 NMDA receptor model showing potential sites for drug action (Kemp and McKernan, 2002)

Over the past decade, a variety of NMDAR subunits have been identified: the ubiquitously express NR1 subunit; a family of four distinct NR2 subunits (A, B, C and D); and two NR3 subunit (A and B) (Cull-Candy, Brickley and Ferrant, 2001; Chatterton et al., 2002)

NR1 and NR2A-NR2D subunits are composed of 938 (main isoform among splice variants), 1464, 1482, 1250 and 1323 amino acids, respectively (Foucaud et al., 2003). NR1 shows low but significant homology (25-28% amino acid sequence identity) with other iGluR subunits and shares a similar hydrophobicity profile to them. Amino acid sequence identities between the NR1 and NR2 subfamilies are as low as 18% and those among the NR2 families are as 40% to 50% (Mori and Mishina, 1995).

3.2 Splice variants

The NR1 subunit gene has a total of 22 exons, three of which (exon 5, 21 and 22) undergo alternative splicing to generate eight NR1 splice variants (Foucaud et al., 2003). Similarly, each of the NR2 and NR3 subunits (apart from NR2A) has several splice variants, although the functional relevance of the different splice forms remains uncertain (Yamakura and Shimoji, 1999; Foucaud et al., 2003).

3.3 Structure

The primary structures of NMDA receptor channel subunits were deduced from their cDNA sequences (Mori and Mishina, 1995). NMDA receptor channel subunits have putative amino-terminal signal peptide and four hydrophobic segments (M1-M4) within their central regions (Stephenson, 2001). The NR2 subunits, especially the NR2A and NR2B subunits, have notably larger carboxyl-terminal domains compared to other glutamate receptor channel subunits (Yamakura and Shimoji, 1999). According to three

transmembrane segment model, segmant M2 forms a reentrant membrane loop with both ends facing the cytoplasm as proposed for the channel-forming pore of voltage-gated ion channels (Wood et al., 1995).

The pattern of water-accessible, exposed residues of the ascending limb of segment M2 is compatible with an alpha-helical conformation which would align exposed residues on one side of the helix (Wood et al., 1995). In contrast, the descending limb may form an extended structure or random coil with consecutive amino acid residues exposed to the lumen of the channel (Wood et al., 1995; Lynch and Guttmann, 2001). The narrow constriction of the channel, which might be located approximately in the middle of the membrane, is considered to result from a cluster of hydrophilic residues situated adjacent to the tip of the descending limb (Yamakura and Shimoji, 1999).

By using reversal potential measurement with organic cations of different sizes to map the diameter of the constriction in wild-type and mutant channels, the asparagine residue at the N site of the NR1 subunit and the asparagine residue at the N+1 site (one position downstream of the carboxyl-terminal side of the N site) of the NR2 subunit were identified as being major determinants of the narrow constriction of the channel which serves as an ion selectivity filter (Wollmuth, 1996).

3.4 Heteromeric Channels

Functional homomeric receptors can be formed with in the AMPA and kainate subunit families but probably not for NMDA receptors (Dingledine et al., 1999). The NR1 subunit forms homomeric channels responsive to L-glutamate plus glycine when expressed in *Xenopus* oocytes, although the current responses are very small (Moriyoshi et al., 1991). When the NR1 subunit is expressed in mammalian expression systems, however, no

functional channels are formed, although specific binding of channels blockers and glycine site antagonists can be detected (Yamamura and Shimoji, 1999).

The involvement of an endogenous *X. laevis* protein would also explain why the NR1 "homomer" are seen *in X. laevis* oocytes and not in mammalian cells (Monyer at al., 1992). The endogenous subunit hypothesis seems to have been generally accepted after it was reported that the mRNA for XenU1, a *X. laevis* glutamate receptor subunit, was expressed at low levels in oocytes (Soloviev and Barnard, 1997). However, the current investigation found that XenU1 does not associate with the NR1 subunit because coinjection of NR1 did not increase the observed currents compared with injection of NR1 alone; similarly, in HEK293 cells, coexpression of XenU1 and NR2 did not result in the formation of functional channels (Green et al., 2002).

Although NR2 subunits do not from functional NMDA receptor channels by themselves, when one of them is coexpressed with NR1, current responses of the heteromeric receptors increase by several orders (Dingledine et al., 1999). Since recombinant heteromeric NMDA receptors display different properties depending on which of the four NR2 or the two of NR3 subunits are assembled with NR1, the NR2 or NR3 subunits can be regarded as modulatory subunits, whereas NR1 serves as a fundamental subunits to form heteromeric NMDA receptors (Ozawa, Kamiya and Tsuzuki, 1998).

3.5 Subunit Stoichiometry

Premkumar and Auerbach (1997) inferred a pentameric stoichiometry for NMDA receptors consisting of three NR1 and two NR2 subunits. An analogous experimental design by Behe found fewer single-channel patterns, however, and concluded there are only two copies of NR1. Concluded that the most parsimonius model involved a tetrameric protein consisting of two NR1 and two NR2 subunits (Behe at al., 1995).

Thus, the conclusion of receptor subunits mixtures are exactly split between a tetramer and a pentamer. Rosenmund et al. and Laube et al. argue that although their data flavor a tetrameric protein, the posibility of a pentameric structure could not be entirely ruled out (Laube, Kuhse and Betz, 1998; Rosenmund, Stern-bach and Steven, 1998). Although the functional results are provocative, an unequivocal determination of the number of subunits in a functional glutamate receptor awaits physical methods that probe the structure of the protein itself (Dingledine et al., 1999; Stephenson, 2001, Vissel et al., 2002).

Little is yet known about the exact subunit composition of native NMDA receptors, but immunoprecipitation straegies have shown that NR2A and NR2B subunits can be coexist together with NR1 in native NMDA receptors gently solubilized from mammalian brain by sodium deoxycholate at pH 9 (Chazot and Stephenson, 1997; Luo et al., 1997).

3.6 Distribution

Spatial and temporal distribution of the NMDA receptor channel subunit mRNAs were examined by *in situ* hybridization analyses (Yamakura and Shimoji, 1999). In the adult rodent, the NR1 mRNA distributed ubiquitously throughout the brain. In contrast, the four NR2 transcripts display distinct regional patterns. The NR2A subunit mRNA is widely distributed in the brain, whereas the NR2B subunit mRNA is predominatly found in the granule cell layer of the cerebellum, while the NR2D subunit mRNA is weakly expressed in the diencephalon and the brain stem (Ozawa et al., 1998; Kemp and McKernan, 2002).

From *in situ* and immunocytochemical analyses, NR3B is expressed predominantly in motor neurons, whereas NR3A is more widely distributed (Chatterton et al., 2002). NR3A is expressed ubiquitously during

development and its expression level reaches a maximum at approximately the first postnatal week. Thereafter, the level gradually decreases, and in adult animals, NR3A is confined to limited nuclei in the thalamus amygdala, and nucleus of the lateral olfactory tract (Nishi et al., 2001).

Expression patterns of the NR2 subunits are also regulated developmentally in rodent brains (Monyer et al., 1992). NR2B and NR2D mRNAs occur prenatally, whereas NR2A and NR2C mRNAs are first detected around birth. The most predominant change is the switch from NR2B to NR2C expression which occurs in the cerebellar granule cells (Ozawa et al., 1998). Since the functional properties of the NMDA receptor channels such as the degree of the voltage-dependent Mg^{2+} block and deactivation kinetics depend on which of the four NR2 is assembled, it is conceivable that different spatial and temporal patterns of expressions of the NR2 genes are designed for fine tuning of NMDA receptor functions in both embryonic and adult brain (Ozawa et al., 1998).

NR1 subunit mRNA is ubiquitously expressed in the brain throughout the different developmental stages (Yamakura and Shimoji, 1999). The NR1 splice variants also display distinct regional and developmental expression patterns (Laurie and Seeburg, 1994).

3.7 Channel Properties

3.7.1 Ca^{2+} permeability

The NMDA receptor channel has characteristic ion permeation properties. The alkali-metal cations, Na^+, K^+, and Cs^+ ions, permeate though the channel with a low selectivity, but major differences from non-NMDA receptor channels exist in the permeation properties for Ca^{2+} and Mg^{2+}. Ca^{2+} is highly permeant, whereas Mg^{2+} is a potent blocker of the NMDA

channel (Ozawa et al., 1998). The resulting Ca^{2+} influx can trigger a variety of intracellular signaling cascades, which can ultimately charge neuronal function though activation of various kinases and phosphatase (Dingledine et al., 1999).

Ca^{2+} ions not only readily pass though NMDA receptor channels but also markedly reduced single channel conductance when the external Ca^{2+} concentration is increased (Jahr and Stevens, 1993). Since this Ca^{2+} block is independent of membrane potential, the Ca^{2+} block site is postulated to be close to the extracellular mouth of the channel pore (Prekumar and Auerbach, 1996). The mutation of the NR1 and NR2A subunit also increases the Ca^{2+} block, and the effects of the mutation seem to be stronger for the NR1 subunit than for the NR2A subunit (Ruppersberg et al., 1993).

3.7.2 Voltage-dependent Mg^{2+} block

The NMDA receptors voltage dependence follows directly from channel block by submillimolar concentrations of extracellular Mg^{2+} rather than from the voltage dependence of conformational changes (Qian and Johnson, 2002). Binding of extracellular Mg^{2+} within the pore is strongly voltage-dependent, and this property dominates the physiological role of NMDA receptors (Dingledine at al., 1999). The block by Mg^{2+} may be explained by assuming that the pore of the channel has a wide mouth located near the extracellular space in which hydrated cations enter easily, and a narrow constriction located deep in the membrane though which only dehydrated Mg^{2+} ions can pass (Dingledine at al., 1999). Since the speed of the replacement of water molecules immediately surrounding the ion is much slower for Mg^{2+} than for other physiological ions (Na^{+}, K^{+}, and Ca^{2+}), the permeant ions. This notion is supported by the fact that Ni^{2+} and Co^{2+}, around which water molecules are replaced as slowly as Mg^{2+}, mimic the effect of Mg^{2+}, but not Cd^{2+}, Sr^{2+}, and Ba^{2+} around which the rate of water exchange is 1000 times faster than Mg^{2+} (Ozawa et al., 1998). More membrane

hyperpolarization would increase the probability that Mg^{2+} occupies the entrance of the constriction region, thereby increasing the degree of the Mg^{2+} block (Dingledine et al., 1999).

NMDA receptor channels are also blocked by physiological concentration of intracellular Mg^{2+} in voltage-dependent manner. The block by intracellular and extracellular Mg^{2+} are suggested to be mediated by different sites of the narrow constriction in the channel (Li-Smerin and Johnson, 1996; Woolmuth, Kuner and Sakmann, 1998).

3.8 Pharmacological Properties

3.8.1 Agonist Binding site

Both NMDA and non-NMDA receptors are activated by the endogenous transmitter, L-glutamate, whereas the putative transmitter candidate, L-aspartate, appears to activate NMDA receptor exclusively (Dingledine et al., 1999).

Johnson and Ascher have demonstrated that NMDA response is markedly potentiated by glycine in cultured central neuron (Johnson and Ascher, 1987). It later turned out that glycine is not simply a strong potentiator of the NMDA response, but is absolutely required for the NMDA receptor channel to enter the open state, thus playing a role as a co-agonist. Although NMDA responses are detectable in nominally glycine-free solution in various preparation, this is due to background contamination of the experimental solutions by glycine (Ozawa, Kamiya and Tsuzuki, 1998). D-serine and D-alanine are activate at the glycine-binding site, and act as co-agonist at the NMDA receptor (Kemp and Leeson, 1993). The ED_{50} value and the extracellular concentration of free D-serine suggest that D-serine also act

as an endogenous co-agonist of the NMDA receptor in the rodent brain (Ozawa et al., 1998).

Binding sites for agonist and co-agonist

Site-directed mutagenesis has identified determinants of glycine binding in distinct regions of the NR1 subunit. The glycine binding site is formed by the region proceeding segment M1 and the loop region between segment M3 and M4 of the NR1 subunit (Yamakura and Shimoji, 1999). In contrast, the glutamate binding site was shown to reside in the homologous regions of the NR2A and NR2B subunits (Anson et al., 1998). Thus, agonist and co-agonist binding sites are located on corresponding regions of distinct subunit of NMDA receptor channels (Yamakura and Shimoji, 1999). In mammalian cells, coexpresion of NR1 and NR2 subunits is essential for forming functional receptors (Ozawa et al., 1998).

All NMDA receptors appear to function as heteromeric assemblies composed of multiple NR1 subunits in combination with at least one type of NR2. The NR3 subunit does not form functional receptors alone, but can co-assemble with NR1/NR2 complexes to produce a functionally distinct triheteromeric NMDA receptor channel (Cull-Candy et al.,2001).

Agonist affinities and antagonist sensitivities of heteromeric NMDA receptor channels are determined by the nature of the NR2 subunit. The ED_{50} values for L-glutamte are 1.7, 0.8, 0.7 and 0.4 μM for the NR1/NR2A, NR1/NR2B, NR1/NR2C and NR1/NR2D subunit channels, respectively (Yamakura and Shimoji, 1999). Diheteromeric NMDARs containing NR2A or NR2B subunits generate "high-conductance" channel openings with a high sensitivity to block by Mg^{2+}, whereas NR2C- or NR2D-containing receptors give rise to "low-conductance" openings with a lower sensitivity to extracellular Mg^{2+} (Misra et al., 2000). Although the

characteristic Ca^{2+} permeability of NMDARs channels is not greatly affected by their NR2 subunit composition (fractional Ca^{2+} current varies between 8-14%), it seems likely that the marked difference in Mg^{2+} sensitivity would affect the Ca^{2+} influx generated by synaptic activation of the different NMDAR subtypes. Recent experiments have shown that the NR3 subunit can also give rise to low-conductance channel opening when co-assembled with NR2A (i.e. NR1A/NR2A/NR3) and these channels shown a roughly five fold reduction in relative Ca^{2+} permeability as compared with NR1/NR2A assemblies (Cull-Candy et al., 2001).

In contrast to NR2 subunits, less is known about the NR3 class of subunits. The NR3A subunit binds to NR1 and NR2 and acts in a dominant negative manner against the NMDA receptor to reduce whole-cell current as well as single-channel conductance (Das at al., 1998), suggesting that the NR3A subunit is important for development and plasticity of the CNS through a modulation of NMDA receptor function (Nishi et al., 2001).

3.8.2 Allosteric modulation site

Proton (pH)

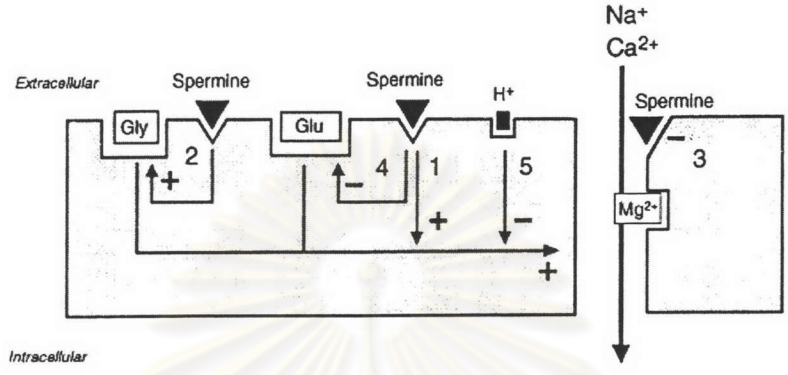
The extracellular pH is highly dynamic mammalian brain and influences the function of a multitude of biochemical processes and proteins, including NMDA receptor function (Dingledine et al., 1999). The NMDA receptor channels are inhibited by protons, with an IC_{50} valure close to physiological pH, implying that NMDA channels are tonically inhibited under normal conditions (Dingledine et al., 1999). The N1 insert of the NR1 subunit controls the proton sensitivities of both the homomeric and heteromeric NMDA receptor channels (Yamakura and Shimoji, 1999). This inhibition occurs primarily through a voltage- and agonist-independent reduction in the

single-channel opening frequency rather than through changes in the single-channel open time or single-channel conductance (Dingledine et al., 1999).

Polyamines

The endogenous polyamines spermidine and spermine are present in high concentration in CNS, and uptake and depolarization induced release of polyamines from brain sliced has been reported (Yamakura and Shimoji, 1999). Spermine have been found to cause block and modulation of a number of types of ion channel (Williams, 1997). Polyamines act on NMDA receptor channels to produce both stimulatory and inhibitory effects by at least four distinct mechanisms.

The multiple effects of spermine on NMDA receptors are summerized in figure 1.2. First, spermine potentiates NMDA currents in the presence of saturating concentrations of glycine (glycine-independent stimulation), an effect that involves an increase in the frequency of the channel opening and decrease in the desensitization of NMDA receptor (Williams, 1997). A second effect involves in increase in the affinity of NMDA receptors for glycine (glycine-dependent stimulation) (Igarashi and Kashiwaki, 2000). A third effect of spermine, a decrease in affinity for glutamate, has been observed at some recombinant NMDA receptors (Igarashi and Kashiwaki, 2000). The mechanism underlying this effect is not known, but it any reflect an increased rate of dissociation of glutamate from the receptor in the presence of spermine. Finally, inhibition by spermine is strongly voltage-dependent and may be due to a fast open-channel block, similar to that by Mg^{2+}. However, the NR1/NR2B channels are not inhibited, but are stimulated by spermine in the presence of extracellular Mg^{2+}. Thus, the voltage-dependent block by spermine



	Homomeric receptors		Heteromeric receptors				
	NR1 + exon 5 (NR1B)	NR1 - exon 5 (NR1A)	NR1A/ NR2A	NR1A/ NR2B	NR1A/ NR2C	NR1A/ NR2D	NR1B/ NR2B
Effect of spermine							
1. "Glycine-independent" stimulation	no	yes	no	yes	no	no	no
2. "Glycine-dependent" stimulation (increase affinity for glycine)	yes	yes	yes	yes	no	no	yes
3. Voltage-dependent inhibition	yes	yes	yes	yes	no	no	yes
4. Decrease affinity for agonists	?	yes	no	yes	no	no	?
5. pH IC_{50}	6.8	7.3	7.2	7.3	6.2	7.3	6.7

Figure 1.2. Subunit-specific modulation of NMDA receptors by polyamines. Four macroscopic effects of spermine have been described that may involve three separate spermine binding sites on NMDA receptors. The effects of spermine are differentially controlled by alternative splicing of exon 5 in the NR1 subunit and by the various NR2 subunits. The effects of protons, which inhibit NMDA receptors, are also controlled in a subunit-dependent manner (Williams, 1997

is likely to be negligible under physiological conditions (Williams, 1997; Igarashi and Kashiwaki, 2000).

Stimulation by spermine is dependent on the type of NR1 splice variant and the type of NR2 subunit present in NMDA receptors. Only receptors expressed from splice variants, such as NR1A, that lack a 21-amino-acid insert encoded by exon 5 show glycine-independent stimulation by spermine (Williams, 1997).

At acidic pH, spermine produces a larger increase in the current response. Since NMDA receptor channels are tonically inhibited at physiological pH, the mechanism of glycine-independent stimulation by spermine may at leasr involve the relief of the tonic proton inhibition (Traynelis et al., 1998).

The NR2B subunit, which forms receptors that are sensitive to stimulation by spermine, is the predominant NR2 subunit in embryonic and neonatal brain (Igarashi and Kashiwaki, 2000). Therefore NMDA rectors in embryonic and neonatal brain may be particularly sensitive to the stimulation effects of polyamines. This is the developmental period of cell proliferation and migration in CNS, and a period when levels of polyamines and activity of ornithine decarboxylase are highly than in adult brain. It is possible that polyamine acting on NMDA receptors could influence neuronal growth, migration and synaptogenesis during development. Excessive activation of NMDA receptor leads to neurodegeneration, and it is conceivable that excessive release of polyamines, for example from injured cells, could exacerbate neuronal injury by potentiating the activity of NMDA receptors (Wiliams, 1997).

Redox agents

Reducing agents such as dithiothreitol (DTT) can potentiate NMDA receptor channels, while oxidizing such as 5-5-dithiobis-2-nitrobenzoic acid (DTNB) are inhibitory (Sucher et al, 1996). The fact that both reducing and oxidizing agent modify native NMDA receptor channels suggests that the redox modulatory sites of the NMDA receptor channel exists in an equilibrium between fully reduced (thiol, R-SH) and fully oxidized (disulphide, R-SS-R) state (Yamakura and Shimoji, 1999). The persistent potentiation by DTT relies on two cysteine residues (cysteine-744 and -798) located in the extracellular loop region between segment M3 and M4 of the NR1 subunit (Sullivan et al., 1994).

It is noteworthy that the two NR1 cysteine residues that control redox modulation also control inhibition by Zn^{2+}, proton and ifenprodil (Dingledine et al., 1999).

Nitric oxide (NO) donors also can inhibit the NMDA receptors, perhaps through the release of NO-derived compounds that support S-nitrosylation of the NMDA receptor (Stamler et al., 1997). However, the exact mechanism of action of NO on NMDA receptors remains controversial (Dingledine et al., 1999).

Zinc

It has been known that group IIB transition metals such as Zn^{2+} and Cd^{2+} inhibit NMDA receptors by both a voltage-dependent and voltage-independent mechanism (Dingledine, et al., 1999). Zn^{2+} also inhibits glutamate uptake and potentiate AMPA receptors, suggesting release of Zn^{2+} might flavor synaptic non-NMDA receptor activation (Johnson, 1998; Dingledine et al., 1999).

Zn^{2+} accumulates at some nerve terminals in specific brain regions and is released into synaptic cleft in Ca^{2+}-dependent manner during neuronal activity (Smart et al., 1994). In cultured neurons, the main effect of Zn^{2+} on the NMDA receptor channels at concentrations as low as 1-10 μM is voltage-independent inhibition (Dingledine et al., 1999). At higher concentrations of 10-100 μM Zn^{2+} additionally produces voltage-dependent inhibition (Yamakura and Shimoji, 1999).

The voltage-independent Zn^{2+}-binding site appears to be strongly dependent on subunit composition, being influenced by NR2 subunit as well as NR1 splice variant (Williams, 1996; Johnson, 1998). Although the concentration of Zn^{2+} in the brain and particularly the synaptic cleft remains a complex question, it is clear that Zn^{2+} can have a multitude of effects on NMDA receptor function (Asher, 1998).

3.9 NMDA receptor antagonists

3.9.1 Competitive antagonists

The classical competitive antagonist of glutamate site on NMDA receptors are phosphono derivatives of short chain (five to seven carbons) amino acids such as AP5 and AP7, whereas halogenated quinoxalinediones and kynurenic acid derivatives were the first competitive glycine site antagonists to be identified (Priestley et al., 1995). More recently, certain phthalazinedione derivatives (Parsons et al., 1997) and benzazepinedione derivatives (Guzikowski et al., 1996) were found highly potent, selective, and systemically active glycine site antagonist.

3.9.2 Noncompetitive antagonists

Several classes of antagonist block NMDA receptors in a voltage-independent manner without causing significantly reduction in agonist potency. Among these, ifenprodil and its analogs have received the most attention (Dingledine et al., 1999). A number of potent and selective NR2B subunit antgonist have since been described, most of which are neuroprotective in animal models and produce minimal side effects at maximally neuroprotective dose (for example, CP-101606, Ro 25-6981 and Ro 63-1908) (Kemp and McKernan, 2002).

3.9.3 Uncompetitive antagonist

An uncompetitive blocker acts only on activated receptor, not the receptor at rest. A general feature of these blocker is that their binding site is made available once the channel is in the open state. It has been speculated that open-channel blockers may be neuroprotective against acute and chronic neurological insults, such as stroke or epilepsy, by limiting the neurotoxic damage of exessive Ca^{2+} entry into cells via NMDA receptors (Dingledine et al., 1999). Early hopes for open-channel blockers such as phencyclidine or MK-801 were disappointed by the appearance of neuropsychiatric and pathological side effect (Deutsch et al., 2003).

4. Treatment of Epilepsy

With the introduction of new and improved therapies, the goal of epilepsy therapy will evolve from achieving seizure control to achieving epilepsy control and enabling the patient to lead a life style consistent with his or her capabilities (Steinhoff et al., 2003). The ultimate goal of therapy will be to target the fundamental cause of the epilepsy syndrome. The success of

epilepsy therapy is dependent on seizure type and the severity of the epilepsy syndrome.

The treatment of epilepsy of all types can be divided into two part: pharmacotherapy and non-pharmacotherapy (Alldredge, 2000).

4.1 Pharmacotherapy

The use of antiepileptic drugs (AEDs) is the most important facet of treatment. Drug therapy provides seizure control in the majority of patients (Decker et al., 2003). In approximately 70% of patients, monotherapy will provide fairly good seizure control and further 10% will be adequately managed with a combination of two drugs. With up to 30% of patients continuing to experience seizure on otherwise optimal treatment a rational basis for the use of all antiepileptic drugs in the management of seizure disorders is required. Mechanism of action may be an important criterion in drug selection process (Kwan and Brodie, 2001).

Twenty years ago, the selection of AEDs, for individual patients relied upon a combination of clinical experience, personal preference and serendipity. Once the diagnosis of epilepsy has been made, the choice of AEDs is guided by considering the relative efficacy and toxicity of each agent (Decker et al., 2003). Proper classification of the patient's seizure type or epilepsy syndrome is the most important step in choosing the appropriate agent (Alldredge, 2000). However, current seizure classification does not define homogenous populations of patients (Decker et al., 2003).

Mechanism of action of antiepileptic drugs

At the cellular level, three major mechanisms of action are recognized; modulation of voltage-gated ion channels, enhancement of γ-

Drugs	Sodium channels	Calcium channels	GABA receptors	GABA synapse	Glutamate receptors
Phenobarbital			+++		
Phenytoin	+++				
Ethosuximide		+++			
Carbamazepine	+++				
Sodium valproate	+	+		++	
Benzodiazepines			+++		
Vigabatrin				+++	
Lamotrigine	+++	++			
Felbamate	++	++	++		++
Gabapentin	+	+		++	
Topiramate	++	++	++	+	++
Tiagabine				+++	
Oxcarbazepine	+++	+			
Levetiracetam	?	?	?	?	?

Key: +++, primary target; ++, probable target; +, possible target; ?, unknown.

Table 1.1 Proposed pharmacological targets of antiepileptic drugs (Kwan and Brodie, 2001)

aminobutyric acid (GABA) mediated inhibitory neurotransmission and attenuation of glutamate mediated excitatory transmission (Kwan and Brodie, 2001).

With the exception of VPA, The established AEDs tend to have clearly defined, single mechanism of action (table 1.1) which facilitates the prediction of effectiveness on the basis of pharmacology (Kwan and Brodie, 2001). It is possible to speculate that partial seizures and primary generalised tonic-clonic seizure respond well to sodium channel blocking drugs (i.e. phenytoin, carbamazepine). In contrast, calcium channel blockers (i.e. ethosuximide) amy be effective against absence seizures and GABAergic agents (i.e. Phenobarbital, Benzodiazepines) and AEDs with multiple mechanisms of action (i.e. Valproate) have utility across a broad spectrum of both partial and generalized seizure disorder (Decker, 2003).

From a pharmacologic point of view, mechanism of action should be an important factor in the rational drug selection process, however it is becoming apparent that mechanisms alone do not adequately predict the success of treatment (Steinhoff et al., 2003). There are a number of possible reasons for this. Firstly, mechanism that matter in individual patients remain unclear. Secondly, treatment of epilepsy targets the symptoms rather than the cause of the disease. Finally, in spite of several clearly defined anticonvulsant mechanism of action, it appears to be an over simplification to suggest that all major modes of action have been discovered at the present time.

In conclusion, it is easy to be dismissive in response to the question of whether mechanisms of action of AEDs can predict success of treatment. However, current evidence suggests that AED pharmacology is of limited utility in the selection of individual drugs for individual patients (Deckers et al., 2003; Steinhoff et al., 2003).

4.2 Non-pharmacotherapy

4.2.1 Vagus nerve stimulation

Vagus nerve stimulation (VNS) is a relatively novel method of treatment for medically intractable epilepsy, introduce in 1988, an used increasingly widely since efficacy and safety were established by clinical trials in the mid-1990s (Binnie, 2000). VNS was approved in 1997 (Alldredge, 2000).

VNS is an empirically based on method for treatment of epilepsy by repeated stimulation of the left vagus nerve through implanted electrodes (Alldredge, 2000). It is a choice available for patients whose partial seizures are poorly controlled as add-on therapy (Cramer, Menachem and French, 2001).

4.2.2 Surgery

Surgery is curative for certain forms of epilepsy. Experience has shown that surgical removal of an epileptogenic lesion in one or other temporal lobe is highly effective (Hopkins, 1993; Jacobs et al., 2001). Patients who are most likely to benefit from surgery are those with partial-onset seizures whose symptoms remain intractable despite optimal medical therapy (Alldredge, 2000).

The optimum treatment for temporal lobe epilepsy, one of the most common forms of drug resistant epilepsy, is no longer controversial. In the first randomized controlled trial comparing surgery with medical treatment. At one year, 58% of surgically treated patients and 8% of medically treated patients were free from seizures, with a number needed to treat of two (Wiebe and Nicolle, 2002).

Surgery is approving with advances in localizing epilepsy substrates by integrated functional and structural mapping prior to, or during, surgery. Sterotactic radiosurgery (gamma knife surgery) has been developed to decrease invasiveness (Jacob et al., 2001).

4.2.3 Alternative therapies

There is, at present, increasing interest in behavioral and other psychological methods of controlling seizure.

Ketogenic diet

The ketogenic diet (KD), introduce in the 1920s, is a high-fat, low carbohydrate, low protein diet (Alldredge, 2000). The KD was used widely before the development of the modern anticonvulsant drugs. As the modern anticonvulsants appeared, however, the KD was used less and less frequently. Recently, however, it has become clear that the KD is often effective against drug-resistant seizures (Thavendiranathan et al., 2003). The KD has, therefore, re-gained popularity as a treatment for intractable epilepsy in infancy and childhood.

Behavioral therapy

Psychological techniques for control of epileptic seizures are often successful for patients with seizures triggered by flashing lights or visual patterns, reading, or listening to music therapies (Alldedge, 2000). In these patients, behavioral therapies in other types of epilepsy remains limited; however, some patients report benefit from relaxation and biofeedback .

5. The development of antiepileptic drugs

Despite over the last two decades, drug therapy for epilepsy has improved substantially (Perucca, 2003), the cellular basis of human epilepsy remains a mystery. In the absence of a specific etiological understanding, approaches to drug therapy of epilepsy must necessarily be directed at the control of symptoms, i. e. the suppression of seizures by chronic administration of antiepileptic (anticonvulsant) drugs (Loscher and Schmidt, 2002; Perucca, 2002). None of the old or new AEDs appears to represent a "cure" for epilepsy or an efficacious means for preventing epilepsy or its progression (Loscher and Schmidt, 2002).

Indeed, the ideal antiepileptic drug would be truly antiepileptic, in addition to being anti-ictal, safe, well-tolerated, convenient to take, devoid of significant drug interactions, and effective when administered for a reasonably short duration (Schachter, 2000).

Current AEDs, although effective in controlling seizures for major of individuals , remain far from ideal as therapeutics (Doherty and Dingledine, 2002). In about 30% of patients with epilepsy the seizure persist despite the choice of an adequate AED and carefully monitored treatment (Regesta and Tanganelli, 1999). Pharmacoresistant epilepsy is a major health problem, associated with increased morbidity and mortality, and accounting the much economic burden of epilepsy (Regesta and Tangenelli, 1999). There is a need for new drugs designed to block the process of epileptogenesis and to reduce the problem of intractable or difficult-to-treat seizures.

Experimental models for antiepileptic drug developments

A diversity of animal models, *in vivo* and *in vitro*, are available for the study of epilepsy and these models have a proven history in advancing

our understanding of basic mechanisms underlying epileptogenesis and have been instrumental in the screening of novel antiepileptic drugs.

The oocytes of the South African clawed frog *X. laevis* are widely used for the expression of heterologous proteins. The functional characterization of membrane proteins related to over excitation in seizure has significantly profited from the use of this expression system.

6. Xenopus oocyte expression system

Oocytes and eggs of the South African clawed frog *Xenopus laevis* have been extensively used in biological and pharmacological research. These cells are excellently suited for investigations on questions of developmental biology, intracellular signaling cascades, biochemical pathways and transport related phenomena (Weber, 1999b). Furthermore, the use of *Xenopus* oocytes for translation of exogenous mRNA was first described by Gurdon and his colleagues in 1971 (Gurdon et al., 1971). Although the *X. laevis* oocyte is endogenously equipped with a host of ion channels, transporter systems and receptors, it has been proven to be an excellent heterologous expression system for the investigation and characterization of countless transport proteins and receptors (Sigel, 1990).

Xenopus laevis is typified by the presence of up to three claws on each of hind limbs of major frog and is a member of the family *Papidae*. The frogs are endemic in South Afruica, Boswana, and Southwest Zimbabwe.

6.1 The biology of the oocyte

6.1.1 Oogenesis and development

Oogenesis in different species is varying, although the functional stages are similar. In *X. laevis* oogenesis, namely growth from stage I to stage VI (Dumont, 1972), is asynchronous, meaning that all stages of growth are usually found in the ovary at a given time. The most obvious differences between oocytes from stage I and stage VI are their size (100 μm and 1300 μm, respectively) and pigmentation which ranges from colorless to marked polarization into the dark animal hemisphere and beige vegetative hemisphere. In stage VI oocytes the two hemispheres are divided by an essentially unpigmented equatorial band. The process of oogenesis requires approximately 8 months (Weber, 1999b).

6.1.2 Bioelectrical properties of the oocyte

For the most electrophysiological, pharmacological and biochemical purposes oocytes of stage V and VI are used. These cells have a diameter of 1.0-1.3 mm and can be easily handled with Pasteur pipettes (Weber, 1999a). Membrane potentials (U_m) of oocytes from a given batch have been shown to be similar, whilst a great variance exists between different batches ranging from –30 to –70 mV, rarely –90 mV can be seen. The oocyte membrane has a high input resistance that differs with oocyte batches and ranges from several 100 kΩ to 2 MΩ and sometimes even more (Fraser and Djamgoz, 1992). U_m is mainly a K^+ diffusion potential, yet the Na^+/K^+-ATPase contributes significantly to U_m (Lafaire and Schwarz, 1986).

6.1.3 Recording

The oocytes system allows performing a whole plethora of different techniques for the investigation of ion channels and transporter. Electrophysiological techniques comprises two-electrode voltage-clamp (TEVC), cut-open technique, patch-clamp in several modes including giant patch, continuous capacitance measurements to study exocytotic and endocytotic process, intracellular ion measurements with selective and other techniques (Wagner et al., 2000).

6.2 Two-electrode-voltage clamp

The two electrode voltage clamp (TEVC) technique is the most widely used electrophysiological technique for the measurement of whole cell currents through ion channels or electrogenic transporters expressed in *Xenopus* oocytes (Wagner et al., 2000). The TEVC allows the control of the membrane potential (clamping) to measure current flowing through ion channels or electrogenic transporter or pumps.

In TEVC recording, two glass microelectrodes are impaled into the oocyte (Weber, 1999b). One intracellular electrode measures the membrane potential (voltage electrode), and the second (current electrode) passes sufficient current to maintain the desired voltage clamp, using a feed back circuit. The amount of current pass through the current electrode is determined by the discrepancy between the membrane potential and the clamping potential. All electrogenic ion or substrate fluxes across the membrane are now measured as a deflection from the base line current.

For the experiments, glass capillaries with a thin filament (to ensure that the filling solution reaches the electrode tip) are pulled, filled with 3M KCl and connected to the feedback amplifier. The resistance of the

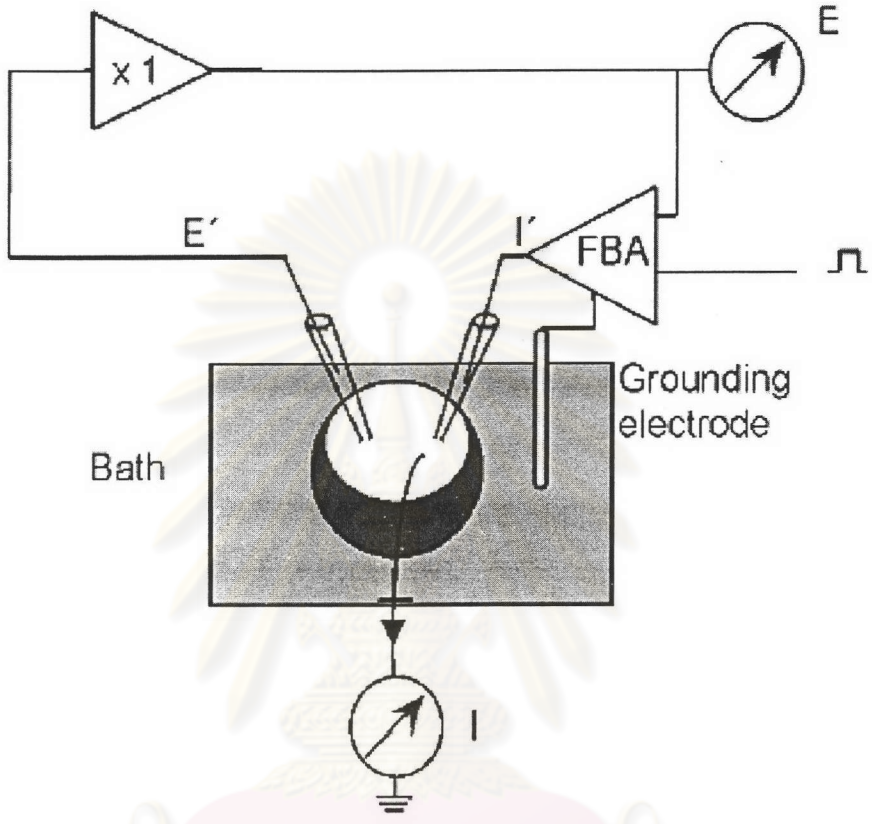


Figure 1.3 Two electrode voltage clamp and its application for *Xenopus* oocytes. TEVC: I' is the electrode delivering the current I needed to clamp the oocyte to the desired membrane potential E which is measured through the voltage electrode E'. FBA is the feedback amplifier measuring current and voltage and delivering the current (Wagner et al., 2000).

electrodes should be around 0.5-5 MΩ. Because of their low resistances, the microelectodes do not clog frequently and hence can be used for several oocytes.

6.3 Advantages and Disadvantages of the oocyte system (Weber, 1999b; Wagner et al., 2000)

Advantages of the Oocyte Expression System

(1) Hundreds of viable cells can be isolated from a given donor frog. The cells can be surgically removed without sacrificing the animal, so one frog can be used several times.

(2) The cells are quite handy and can survive for up to 2 weeks in vitro. The cells can tolerated impalements of microelectrodes and injection pipettes. Moreover, relatively simple facilities are required for maintaining the cells, once isolated.

(3) The cells are big (up to 1.3 mm in diameter) and can be easily injected with DNA, RNA, as well as membrane-impermeable drugs.

(4) The oocytes faithfully express foreign RNA that has been injected into them.

(5) The oocytes has only a few endogenous channels (the major one being a Ca^{2+} activated Cl^- channel), which usually carry only a small fraction of the current expressed. This permits a particular channel to be studied in virtual isolation.

(6) Macropatch recording are possible in oocytes. These enable low-noise, fast-clamp patch recordings of many channels that can not be obtained using other expression systems.

(7) Expression cloning into into oocytes greatly speeds up the purification and isolation of RNA for a desired protein. Separating total brain RNA, for example, in fractions on the basis of size followed by injection of

each fraction enables the rapid location of the protein's RNA to a particular band size.

Disadvantages of the Oocyte Expression System

(1) Because of its large size, whole-cell patch-clamp experiments, where one can control the intracellular ionic composition by dialysis across the patch pipettte, are not possible.

(2) The endogenous channels, although few, can interfere with current measurements if they are small (e.g., gating charge or mutants having little expression).

(3) Posttranslational modifications may be different in oocyte compared with the native cells. Hence, channels may actually function differently in their native environment.

(4) In some laboratories, oocytes exhibit seasonal variation such that channel expression and ability to obtain seals are more difficult in the summer months.

(5) It should be borne in mind that *Xenopus* is an amphibian, and the cells should only be studied at room temperature (18-22°C). At higher temperatuires, the cells rapidly deteriorate. Most channels and receptors that are expressed in the oocyte are of mammalian origin. Since certain processes depend critically on temperature (oscillationa in cytosolic free Ca^{+}, rates of activation and inactivation of channels), experiments on oocytes may not be physiological significance with respect to the cells from which the protein is derived.

7. Valproic acid

Valproic acid (VPA) or valproate is the trivial name for 2-propylpentanoic acid. Since its first marketing as an antiepileptic drug (AED) 35 years ago in France, valproate has become established worldwide as one of

the most widely used AEDs in the treatment of both generalized and partial seizures in adults and children (Loscher, 2002).

7.1 Mechanism of action

Because of its wide spectrum of anticonvulsant activity against different seizure types, valproate has repeatedly been suggested that it acts through a combination of several mechanisms (Perrucca, 2002). Valproate increases GABA synthesis and release and thereby potentiate GABAergic functions in some specific brain regions, such as substantia nigra, thought to be involved in the control of seizure generation and propagation (Loscher, 2002). Furthermore, valproate seem to reduce the release of the epileptogenic amino acid δ-hydroxybutyric acid. Microdialysis data suggest that valproate alters dopaminergic and serotoninergic function (Loscher, 2002).

7.2 Effects on NMDA receptor functions

Experimentals on mouse neurons in culture indicated that neuronal responses to excitatory amino acid such as glutamate, as not altered by valproate at relevant concentrations (Loscher, 1998). However, several studies showed direct actions on NMDA receptors:

(1) Blocking neuronal firing induced by NMDA receptor activation (Loscher, 1998).

(2) Suppression of NMDA evoked transient depolarisation in rat neocortical pyramidal cells (Zeise, Kasparaow and Zeiglgansberger, 1991). VPA has no effects on membrane responses mediated by kainate- or quisqualate-sensitive receptors.

(3) Decrease in NMDA receptor mediated synaptic responses in rat amygdala slices (Gean et al., 1994).

(4) Decrease in NMDA induced excitatory post-synaptic potentials in rat hippocampus (Ko, Brown-Croyts and Teyler, 1997).

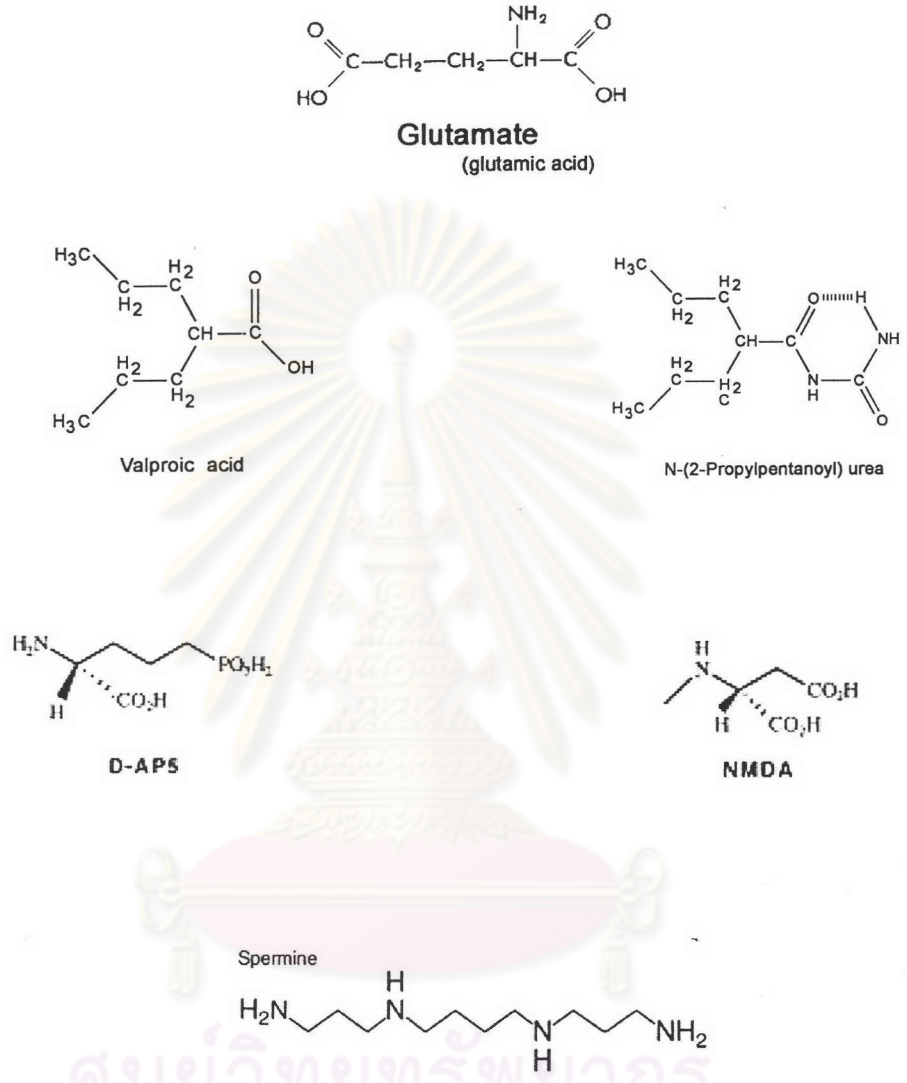


Figure1.4 Molecular structure of glutamate, NMDA, AP5, spermine, valproic acid and VPU

(5) Effects on neuronal conductance when fiber/synapes are functioning abnormally. In vitro VPA limits high frequency sustained repetitive firing of Na^+ dependent action potentials through blockade of voltage dependent Na^+ channels (McLean and McDonald, 1986).

(6) VPA suppresses spontaneous epileptiform activity in hippocampal slices by activation of Ca^{2+} dependent K^+ conductance (Franceschetti, Hamon and Heineman, 1986).

Although the GABAergic potentiation and glutamate/NMDA inhibition could be a likely explanation for the anticonvulsant action on focal and generalised convulsive seizures, they do not explain the effect of valproate on nonconvulsive seizures, such as absences (Loscher, 2002). In this respect, the reduction of gamma-hydroxybutyrate (GHB) release reported for valproate could be of interest, because GHB has been suggested to play a critical role in the modulation of absence seizures. Although it is often proposed that blockade of voltage-dependent sodium currents is an important mechanism of antiepileptic action of valproate, the exact role played by this mechanism of action at therapeutically relevant concentrations in the mammalian brain is not clearly elucidated (Loscher, 2002; Perucca, 2002). In view of the diverse molecular and cellular events that underlie different seizure types, the combination of several neurochemical and neurophysiological mechanisms in a single drug molecule might explain the broad antiepileptic efficacy of valproate (Loscher, 2002).

7.3 Side effects

The incidence of toxicity associated with the clinical use of VPA is low, but two rare toxic effects idiosyncratic fatal hepatotoxicity and teratogenicity, necessitate precautions in risk patient populations (Loscher, 1998). The risk of hepatotoxic failure from VPA in a polypharmacy regimen is

1 in 500 patients under the age of 2, as compared < 1 in 12,000 in older patients treated with polypharmacy (Johannessen and Johannessen, 2003).

8. N-(2-Propylpentanoyl)Urea (VPU)

Because the need for new drug is clear, the search for more effective and safer AEDs continues. A number of valproate derivatives were synthesized and evaluated the anticonvulsant and neurotoxic effects.

N-(2-Propylpentanoyl)Urea (Valproyl urea, VPU) is a new synthetic derivative of valproic acid which was synthesized by Boonardt Saisorn and co-worker (1992). Anticonvulsant activity of VPU was previously investigated by a number of investigators (Sooksawate, 1995; Chunngam, 1996; Tantisira et al., 1997). They have found that VPU can protects mice against the maximal electroshock (MES) test and the pentylentetrazole (PTZ) test. In addition, it is also orally active and seems to offer a greater safety margin in pararellel with lower unwanted effects in relation to its parent compound, valproate.

To search for the mechanism of action of VPU, a variety of *in vivo* and *in vitro* models were used. In brain microdialysis studies on anesthesized rats, VPU decreased the level of cortical excitatory (aspartate and glutamate) and inhibitory (glycine and GABA) amino acid neurotransmitter (Sooksawate, 1995). However, in awake rat, VPU was found to exert no significant effect on the level of cortical aspartate, glycine and GABA while a non dose-dependent reduction was observed on glutamate level (Chunngam, 1996). In previous study on neurons of rat cerebral cortex and cerebellar Purkinje cells by microiontophoretic techniques, VPU depressed spontaneous firing of both neurons of cerebral cortex and Purkinje cells (Khongsombat, 1997). At present, the exact mechanism of action of VPU is not clearly elucidated.

With regards to two major adverse effects of VPA, embryotoxicity and hepatotoxicity, the effects on axial rotation and embryonic growth were lower in VPU-treated animals compared with those of VPA-treated animals (Meesomboon et al., 1997). Studies in rats and isolated rat hepatocytes demonstrated that VPU was safer than VPA as hepatotoxicity of VPU was observed when very high dose was given (Patchamart, 1996). Pharmacokinetic studies utilizing ^{14}C-VPU and autoradiographic technique demonstrated a rapid distribution characteristic of VPU into various organ tissues. In addition, *in vitro* studies using carboxylesterase from human liver and phenobarbital-treated mice liver showed that VPU was negligibly hydrolysed into VPA. Therefore, it was postulated that VPU and/or any metabolites other than VPA was responsible for the anticonvulsant activity.

9. Objectives of the present research

VPU, a monoreide analogue, has the same aliphatic side chain as valproic acid. This compound exhibited a good prospect of being a potent broad spectrum antiepileptic drug with higher margin of safety and lower side effect than its parent compound. Mechanism of action of VPU is important in process of drug development and will be useful to explain and evaluate the efficiency of this compound.

The NMDA receptor is an important target for AED development, with agents from many different classes acting on this receptor. The purpose of this study was to investigate the effect of VPU on NMDA receptor using the *Xenopus* oocyte expression system. The oocytes injected with the mRNAs of NR1A and NR2B sunbunits expressed functional NMDA receptor and were recorded with TEVC technique, whole cell recording technique.