CHAPTER IV

DISCUSSIONS AND CONCLUSIONS

Inappropriate activation of NMDA receptors has been implicated in the aetiology of several disease states (Cull-candy et al., 2001). In theory, any disorder caused by glutamate-induced excitatory has the potential to be treated by blocking of NMDA receptors (Kemp and McKernan, 2002). In particular, epilepsy, in which there is overactivity of excitatory pathway, would be candidate for NMDA receptor antagonist treatment.

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 et al., 1998).

Although only a single NR1 gene has been identified, the NR1 subunit can potentially exist in eight different splice variants, based upon alternative mRNA splicings at each of three splice sites (Dingledine et al., 1999). The NR1 transcript is found throughout the rat brain (Standaert et al., 1994). Four NR2 genes, NR2A-D, have been identified (Katsuwada et al., 1992). NR1A/NR2A and NR1A/NR2B are the most widely expressed NMDA receptor subtypes in the central nervous system (Mori and Mishina, 1995) and are believed to correspond to `antagonist-' and `agonist-preferring' NMDA receptors (Laurie and Seeburg, 1994).

However, the subunit specificity, and the effect of seizure activity on subsequent receptor expression and function are not yet fully understood, Narita et al. (2000) suggested that the NR1, NR2A and NR2B subunits may involved in seizure sensitivity to glutamatergic agonists and consequently have an important role in regulating seizure activity. Moreover, tyrosine

phosphorylation of NR2B by Fyn (Transgenic mice) might be involved in the susceptibility to NMDA receptor-mediated kindling (Kojima et al., 1998).

In the present study, VPU was found to exert significant inhibitory effects on NMDA receptor currents elicited by the application of glutamate to *Xenopus* oocyte expression system expressing NR1A/NR2B NMDA receptor sutype.

Such inhibition could be accounted by modulation of several binding sites on NMDA receptors such as : (1) agonist recognition site binding, (2) co-agonist recognition site binding, (3) modulatory site binding, (4) open-channel blocking and (5) the other sites binding such as selective antagonist site. Thus, inhibitory effects of VPU on glutamate-induced currents in various conditions were further examined in *Xenopus* oocytes injected with NR1A/NR2B mRNAs.

VPU (1-300 μM) *per se* produced no changes of the membrane conductance in oocytes with or without an injection of NR1A/NR2B mRNAs, indicating that VPU could not directly activate any ion channels including NMDA receptor that are intrinsically or exogenously expressed in the oocyte membrane. It has been reported that stimulation of NMDA receptors expressed in *Xenopus* oocytes activates Ca^{2+} channels coupled with the receptors as well as the oocytes's intrinsic Ca^{2+}-dependent Cl^- channels (Kelso et al., 1992). Thus, it is likely that inhibitory effect of VPU on NMDA responses might resulted from blocking of Ca^{2+}-dependent Cl^- channels. However, this possibility was ruled out by the analysis of the voltage-and-current relationship in terms of glutamate activation of NMDA receptor in the presence and absence of VPU which were reversed at around 0 mV, potential that is closer to the reversal potential for the NMDA channels in neurons (Mayer and Westbrook, 1987).

In the present study, the glutamate dose–response curve was shifted to the right and in a parallel fashion in the presence of VPU, however, the maximal glutamate response could be obtained even in the presence of high concentrations of VPU. Together, these data are consistent with the notion that VPU acts as a competitive antagonist of the NMDA receptor at glutamate recognition sites.

VPA, parent compound of VPU, was also tested with the same conditions. Although, several studies have demonstrated the inhibitory effects of VPA on NMDA receptors, however, in the present study high dose (1mM) of VPA could not show any significant effects on glutamate-induced currents. Discrepancy of the results may be due to the selective properties of the VPA inhibition on NMDA receptor subtypes.

Antagonism of NR1A/2B receptors by VPU is independent of membrane voltage, which argues that VPU is not acting at a site deep in the channel pore (Huttner and Bean, 1988). Taken together with a result in figure 3.4 - 3.7, inhibition of NMDA receptor function by VPU would appear to be mediated by a glutamate site located on the external surface of the receptor.

The effects of VPU on NR1A/NR2B NMDA receptor at glycine coagonist site were also investigated. Based on the finding that VPU produced no significant change in the maximal response and slope coefficient of glutamate dose-response curve in the presence of various concentration of glycine, the possibility that VPU would competitively bind at co-agonist site was ruled out.

Previous studies has demonstrated that glycine site antagonists displayed the selectivity for NMDA receptor. Glycine has been shown to have a lower affinity for the NR1A/NR2A than the NR1A/NR2B receptor (Kasuwada et al., 1992). Competitive antagonists acting at this site might

displace glycine preferentially from the NR1A/NR2A receptor and be, consequently, more potent antagonists at this receptor subtype. Thus, the effects of VPU at glycine site of NR1A/NR2A receptor subtype in the further study should be investigated further.

Recombinant NMDA-receptor currents were very weakly antagonized by VPU. When inhibitory effects of VPU on glutamate-induced currents was compared to those induced by AP5, a well-characterized NMDA receptor antagonist, it is apparent that the inhibitory effect of VPU was rather weak. This finding indicates that blocking effects of VPU on NR1A/NR2B receptors could not be the major mechanism of action of VPU which underlies anticonvulsant activity of VPU in various animal models.

The modulatory effects of spermine on NMDA receptors involve site on the extracellular domains of the receptor (Williams, 1997). In these studies, the effects of co-application of VPU and spermine on NMDA receptor currents were dependent on the concentration of VPU. The stimulatory effect of spermine disappeared at high concentration of VPU. Therefore, It demonstrated that VPU counteracts effects of spermine in competitive manner.

Previous elctrophysiological studies with NR1A/NR2B expressing oocytes demonstrated that spermine reduced glutamate potency that would thus increase the apparent affinity of competitive antagonists (Donevan and McCabe, 2000). Although, increasing inhibitory effects were not shown in this study, the percentages of inhibitions of VPU are increased significantly in the presence of spermine. Polyamines are released in a calcium dependent-manner from neurons in response to chemical, electrical, or K^+ stimulation (Donevan and McCabe, 2000). Moreover, seizures and ischemia have been associated with an increase in polyamine levels (Hayashi et al., 1993). The activity-dependent release of spermines may enhance inhibitory effects of VPU during periods of intense neuronal activity such as that occurring during seizures.

In addition to the inhibitory effect of VPU on NMDA receptors, previous studies demonstrated the effect of VPU on the $GABA_A$ receptor and that VPU may have some interaction directly or indirectly with the barbiturate site(s) on the $GABA_A$ receptor (Chanthes, 2002). Together, these data may explain the broad anticonvulsant activities of VPU in rather many seizure models in animals.

In conclusion, the data presented here suggest that VPU is a competitive inhibitor of NR1A/NR2B receptor. The shift of glutamate EC_{50} for NMDA receptor and an increasing of inhibitory effects in the presence of spermine support a competitive mechanism. Furthermore, these studies showed that blocking effects of VPU are glycine- and voltage-independent. Its effects on NMDA receptor is not potent and seems not to be the principal mechanism of action underlying anticonvulsant effects in *in vivo* models. However, the finding that inhibitory effect of VPU was markedly potentiated when being co-application with spermine indicating that its potency may be increase in seizure conditions. Therefore, in addition to interaction at $GABA_A$ receptor as previously reported, the inhibitory effects of VPU on NR1A/NR2B receptor may, at least in part, contributes to its mechanism of anticonvulsant action.