Role of Altered Structure and Function of NMDA Receptors in Development of Alcohol Dependence

József Nagy*, Sándor Kolok, András Boros, Péter Dezső

Gedeon Richter Ltd., Pharmacological and Drug Safety Research, Budapest 10. P.O.Box 27, H-1475, Hungary

Abstract: Long-term alcohol exposure gives rise to development of physical dependence on alcohol in consequence of changes in certain neurotransmitter functions. Accumulating evidence suggests that the glutamatergic neurotransmitter system, especially the N-methyl-D-aspartate (NMDA) type of glutamate receptors is a particularly important site of ethanol's action, since ethanol is a potent inhibitor of the NMDA receptors (NMDARs) and prolonged ethanol exposition leads to a compensatory "upregulation" of NMDAR mediated functions supposedly contributing to the occurrence of ethanol tolerance, dependence as well as the acute and delayed signs of ethanol withdrawal.

Recently, expression of different types of NMDAR subunits was found altered after long-term ethanol exposure. Especially, the expression of the NR2B and certain splice variant forms of the NR1 subunits were increased in primary neuronal cultures treated intermittently with ethanol. Since NMDA ion channels with such an altered subunit composition have increased permeability for calcium ions, increased agonist sensitivity, and relatively slow closing kinetics, the above-mentioned alterations may underlie the enhanced NMDAR activation observed after long-term ethanol exposure. In accordance with these changes, the inhibitory potential of NR2B subunit-selective NMDAR antagonists is also increased, demonstrating excellent potency against alcohol withdrawal-induced *in vitro* cytotoxicity. Although *in vivo* data are few with these compounds, according to the effectiveness of the classic NMDAR antagonists in attenuation, not only the physical symptoms,but also some affective and motivational components of alcohol withdrawal, novel NR2B subunit selective NMDAR antagonists may offer a preferable alternative in the pharmacotherapy of alcohol dependence.

Key Words: Alcohol, dependence, withdrawal, NMDA receptor, NR2B subunit selective antagonist, pharmacotherapy.

INTRODUCTION

According to the recent developments in drug abuse research across the areas of molecular genetics, cell biology, animal behaviour, and human brain imaging studies, the widely held elderly view about drug abuse and addiction – i.e. that the problem of drug abuse would go away if only the abuser or addict could change his behaviour – tends to be unmaintainable. Recent advances in our understanding of the neurobiology of drug abuse highlight the importance of the interpretation of drug addiction as a complex brain disease caused by alterations in crucial neurotransmitter systems, and thus give rise to the opportunity of pharmacological interventions [78].

Drug dependence (American Psychiatric Association, 1994) [6], also termed as drug addiction, is a chronically deteriorating disorder characterised by the desire to seek for and take the drug, leading to the loss of control in limiting intake because of the emergence of psychical and/or physical dependence, i.e. a negative emotional and/or a disturbed physiological state, when access to the drug is withdrawn. These behavioural and/or physiological abnormalities develop gradually and progressively during a course of repeated exposure to a drug of abuse. According to the recent view, drug dependence can be considered as a form of drug-induced neural plasticity. Repeated exposure to a drug of abuse alters the amounts, and even the types of genes expressed in specific brain regions. The altered expression of genes then mediates altered functions of individual neurons as well as the neural circuits within which the neurons operate. Finally, such changes in the neural circuit underlie the abnormalities seen in a drug dependent person (Fig. **1**) [104, 159, 161, 92, 120, 157, 160].

Among the numerous types of drug addictions, alcohol use disorders represent a substantial public health problem all over the world. In 2000, 2 billion alcohol users were estimated by the World Health Organisation (WHO), compared with 1.3 billion smokers and 185 million users of other psychoactive drugs. According to the data released by the WHO in 2003, the global prevalence of alcohol use disorders was 1.7%, and these disorders accounted for 1.4% of the total world disease burden. Data released by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) in August 2004 showed that whereas in 1991–1992, the total prevalence of 12-month alcohol abuse and dependence, according to the "Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition" (DSM-IV), was 7.41% in the USA representing 13.8 million adult Americans, this prevalence rose to 8.46% representing 17.6 million Americans in 2001–2002. Given the harmful effects of alcohol use disorder on the afflicted individuals and society as a whole, alcohol use disorders continue to represent one of the world's major health problems, with large direct health costs (psychiatric and physical) as well as massive indirect costs to society in terms of crime, loss of earnings and productivity, and social damage [72, 134].

In alcohol dependent individuals a complex set of signs, i.e. alcohol withdrawal syndrome (AWS) occurs after

*Address correspondence to this author at Gedeon Richter Ltd., Pharmacological and Drug Safety Research, Budapest 10. P.O.Box 27, H-1475, Hungary; Fax: 36-1-2605000; E-mail: jozsef.nagy@richter.hu

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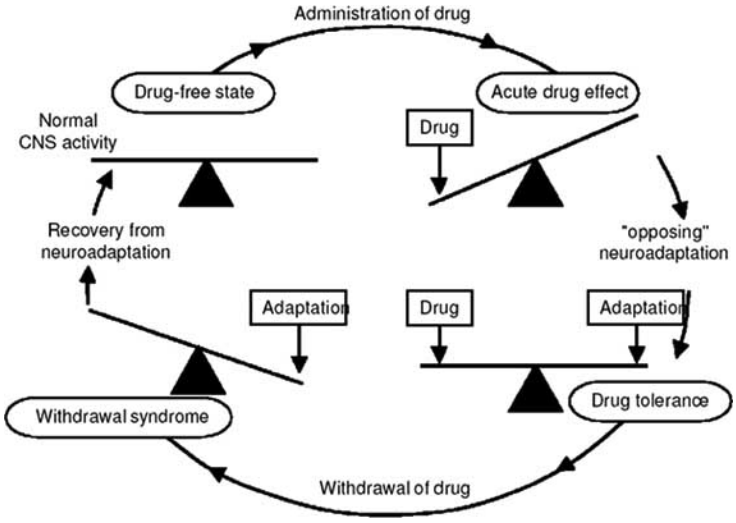

Fig. (1). Drug dependence as neuroadaptation.

The concept is that the administration of a drug acutely "unbalances" the chemistry of the brain. In order to overcome this effect, the brain institutes a homeostatic mechanism, i.e. an "opposing neuroadaptation" that balances the effect of the drug on brain chemistry. While the drug is present in the brain, the system remains in relative balance (i.e. there is evidence of drug tolerance). However, rapid removal of the drug now exposes the adaptation because it is no longer "balanced" by the drug. The resulting functional disturbance is the cause of the drug withdrawal syndrome. In Himmelsbach's theory, this will continue until the adaptation can be removed and the chemistry of the brain returns to its normal balancing act. Collier's modification of this hypothesis was to propose that, since drugs act on receptors in the brain, it was logical to suppose that a primary mechanism for neuroadaptation to drugs would be to regulate the numbers of those receptors. This type of adaptation would reduce the effects of the drug, but would also cause alterations when the drug left the brain because the natural transmitters inside the brain also use the same receptors. This modified unitary, hypothesis remains implicitly accepted by neuropharmacologists today, but we are beginning to recognize that it represents a gross oversimplification of the complex cellular mechanisms for drug dependence.

From: John Littleton. (2001) Receptor regulation as a unitary mechanism for drug tolerance and physical dependence—not quite as simple as it seemed! *Addiction* **96**, 87–101.

alcohol cessation. The symptoms of the withdrawal syndrome include sweating, tremor, hypertension, anxiety, agitation and sympathetic hyperactivity responsible for tachycardia within the first hours following the last alcohol intake. Later on epileptic seizures and delirium tremens characterized by auditory and visual hallucinations, confusion and disorientation, clouding of consciousness and pronounced autonomic hyperactivity may also occur. This set of symptoms can even lead to death from respiratory and cardiovascular collapse. Even minor symptoms are disabling enough to lead the alcohol dependent individual to resume alcohol consumption at the early stages of withdrawal. The severity of alcohol withdrawal syndrome is therefore a major risk factor for early relapse [216].

Until recently, little could be done to help problem drinkers' control over alcohol consumption. Thanks, however, to discoveries on the neurochemistry of alcohol's effects and on the complex pathophysiology of withdrawal symptoms, some biology-based treatments are now available and even more help is on the way. The research is leading to an increased understanding of how various neurotransmitter systems in the brain contribute to the development of alcohol dependence, thus a wider range of treatment options may arise for individuals with alcohol problems [11]. This review focuses on the role of a special type of glutamate receptors, the N-methyl-D-aspartate receptors in the development of alcohol dependence and in possible therapeutic approaches.

ETHANOL AND NMDA RECEPTORS

Although, the exact mechanism by which ethanol exerts its effect is still a matter of debate, a major step in medication development occurred in recent years when scientists discovered evidence that alcohol acts on several chemical systems in the brain to create its alluring effects. Besides its well-known effect on the release of neurotransmitters especially dopamine (DA), resulting in increased DA levels in the mesolimbic system including the nucleus accumbens, it is now clear that ethanol also alters the function of a number of neurotransmitter receptors (e.g. γ-amino butyric acid A ($GABA_A$), glycine, glutamate, norepinephrine, DA, serotonin, acetylcholine and opiate receptors), as well as transporters (adenosine, norepinephrine, DA, serotonin transporters). Particularly, ion channels including the L-type voltage-gated Ca^{2+} channels (VGCC) [127] and the ligand-gated channels of the main amino acid neurotransmitter systems of the brain – the inhibitory GABA and the excitatory glutamate – seem to be highly sensitive to the acute effect of ethanol at relevant (5 – 100 mM) concentrations [26, 63, 70, 118, 123].

In the past years, there has been increasing evidence that acute ethanol facilitates GABAergic transmission by enhancing chloride conductance through the $GABA_A$ receptors, and inhibits glutamatergic function by decreasing cationic conductance through the ionotropic (i.e. receptors containing a ligand-gated ion channel) type of glutamate

receptors. Conversely, long-term ethanol exposure appears to create inverse changes in the functions of these systems leading to decreased GABAergic and increased glutamatergic functions bringing about the development of tolerance and/or dependence as well as withdrawal symptoms when alcohol use is cut off [120]. According to the recent results on cell and animal studies, the glutamatergic system is considered as an especially important factor in the mediation of the addictive effect of alcohol [48]. Among the family of ionotropic glutamate receptors including the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), the kainate, and the NMDA receptors, the AMPA and particularly the NMDA type of receptors represent the highest affinity targets for ethanol in the CNS [70, 82, 110, 122].

Structure and Function of NMDA Receptors

NMDA receptors (NMDARs), like other ion-channel receptors, appear to be multimeric transmembrane proteins, composed of different types of subunits. The ubiquitously expressed NR1 subunits exist in eight distinct isoforms (depending on the inclusion or exclusion of the N1, C1, and C2 or C2' cassettes) because of three independent sites of alternative splicing. Four different subtypes of NR2 (A, B, C and D) and two subtypes of NR3 (A, B) subunits are also identified [47, 84, 141]. Although, the precise subunit composition and stoichiometry of native NMDARs are difficult to determine, NMDARs are believed to exist as tetrameric complexes consisting of at least one NR1 and one NR2 subunits [114, 139, 140, 141, 172]. The subunits are most probably arranged as dimer of dimers with an NR1-NR1-NR2-NR2 orientation in the channel [189]. Each subunit has four hydrophobic regions, although only three of them form membrane-spanning domains (TM1, TM3, and TM4). The fourth one (M2) makes a hairpin bend within the membrane and participates in the formation of the ion channel [13, 45] (Fig. **2**).

The involvement of NMDARs in diverse processes like excitatory synaptic transmission [205], synaptic plasticity [127], neurotrophic and neurotoxic functions [102, 163, 185] rests upon their unique features, i.e. i) their high permeability to Ca^{2+} ions, ii) their relatively slow activation/deactivation kinetics, and iii) their voltage-sensitive blockage by extracellular Mg^{2+} ions. Glutamate, the native agonist of the NMDARs, can open the ion-channel only if the plasma membrane became depolarised and the Mg^{2+} blockage was displaced. Thus, NMDARs act as coincidence perceptive elements, which become active only when electrical and chemical signals are present concurrently.

Besides glutamate, NMDARs are sensitive to several other endogenous modulators including their co-agonist glycine [135] and D-serine [144]. Endogenous polyamines, spermine and spermidine also facilitate [115, 180], whereas extracellular Zn^{2+} ions [37] and protons [202, 206] suppress NMDAR activation. NMDARs interact with various intracellular scaffolding, anchoring, and signalling molecules associated with the postsynaptic density (Fig. **2**, see review of [121]). The sensitivity of NMDARs to different ligands, its permeation, and block by divalent ions, kinetic properties, and interaction with intracellular proteins highly depend on their subunit composition [21, 39, 91]. Diheteromeric NMDARs composed of NR1/NR2A or NR1/NR2B subunits generate 'high-conductance', Mg^{2+} sensitive channels permeable also to Ca^{2+} ions. On the contrary, receptors containing NR2C or NR2D subunits give rise to 'low-conductance' channels with a lower sensitivity to Mg^{2+} ions

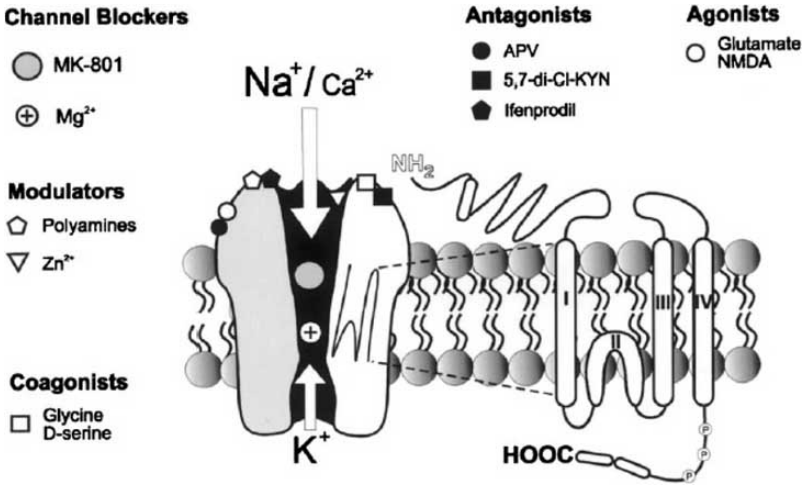

Fig. (2). Schematic diagram of NMDA receptor ion channel.

Diagram representing NMDA receptor ion channel with its various regulatory sites. The receptor is activated by agonists such as glutamate or NMDA. APV is a competitive antagonist, 5,7-di-Cl-KYN binds to a strychnine insensitive glycine site, ifenprodil is a polyamine site antagonist. The open NMDA channel is blocked by Mg^{2+} and by uncompetitive antagonists such as MK-801. Glycine and D-serine act as coagonists. Additionally, polyamines and Zn^{2+} ions modulate the NMDA receptor. There are phosphorylation sites (P) that modulate responses of the receptor to agonists and may play a role in synaptic plasticity. Each subunit is believed to have four regions (I, II, III, and IV) within the cell membrane

From: Bisaga, A. and Popik, P. (2000) In search of a new pharmacological treatment for drug and alcohol addiction: N-methyl-D-aspartate (NMDA) antagonists. *Drug Alcohol Depend.* **59**, 1–15.

and permeability principally to Na^+ ions. The NR3 subunits are thought to be regulatory in nature, since they do not form functional channels with NR1 subunits but co-assemble with NR1/NR2 complexes forming 'low-conductance' channels [168].

Ethanol is a Potent Inhibitor of NMDA Receptors

Biochemical, electrophysiological and behavioural evidences show that ethanol - in clinically relevant concentrations (25 – 100 mM) - is a potent and selective inhibitor of NMDA receptors [50, 81, 122, 124]. Several studies involving recombinant receptors have demonstrated that receptors containing different types of NR2 subunits have differential sensitivity to the inhibitory effect of ethanol [170, 192]. According to the earlier results, the ability of ethanol to depress NMDA evoked currents paralleled with the neuroprotective action of ifenprodil in rat cultured cortical neurons [125]. Similar results were obtained when NMDA-induced release of (^{3}H)-norepinephrine was measured in slices from cerebral cortex of the rat [58]. Since ifenprodil is known as an NR2B subunit selective NMDAR antagonist [213], it was assumed that ethanol acts on the same subunit. Indeed, studies performed on recombinant NMDARs showed that heteromers containing either NR2A or NR2B subunits are preferentially sensitive to ethanol inhibition *vs*. heteromers containing NR2C or NR2D subunits [24, 38, 112, 131, 136, 215, 219]. Moreover, NMDARs with NR1/NR2B subunit combination were more susceptible to the effect of ethanol compared to those composed of NR1/NR2A subunits [7, 14, 15, 192]. The co-expression of NR3 or an NR3-GFP fusion protein with NR1/NR2 (A-D) subunits did not alter the inhibitory effects of ethanol [193].

Data regarding the site of effect of ethanol on NMDARs are controversial. Earlier it was thought that ethanol binds to a hydrophobic pocket distinct from other modulatory binding sites of the NMDARs [166, 167]. Recently it was suggested that this pocket is associated with the third transmembrane domain (TM3) of the NR1 subunit [181]. While mutation of Phe639 to Ala in this region of the NR1 subunit expressed in either oocytes or HEK-293 cells significantly decreased the inhibitory effect of ethanol, the substitution of this residue for Trp resulted in receptors that were slightly more sensitive to ethanol inhibition than the wild-type receptors. These observations suggest that the 639 position of the NR1 subunit is an important determinant of ethanol sensitivity [2].

Action of ethanol on NMDARs may also be mediated by changes in the phosphorylation status of the receptor subunits. According to Alvestad *et al*. [5], phosphorylation of tyrosine side chains in the NR2A and/or NR2B subunits was significantly reduced following *in situ* exposure of hippocampal slices to 100 mM ethanol. Addition of a phosphotyrosine phosphatase inhibitor – bpV(phen) – in the recording medium prior to and during ethanol exposure significantly reduced the inhibitory effect of ethanol on NMDAR mediated excitatory postsynaptic potentials [5, 54]. These data suggest a possible mechanism by which ethanol may inhibit NMDAR functions *via* activation of a tyrosine phosphatase and phosphatase-mediated dephosphorylation of NMDAR subunits may play a role in mediating the inhibitory effect of ethanol.

Effect of Long-Term Ethanol Exposure on NMDAR Functions

Data from studies on neuroadaptation following long-term ethanol exposure indicate a significant role of NMDARs in the development of alcohol dependence, in the expression of alcohol withdrawal syndrome as well as in withdrawal associated neuronal damage. Initial *in vivo* studies showed that seizures evoked by withdrawal of ethanol in alcohol dependent animals were attenuated by NMDAR antagonists and exacerbated by administration of NMDA at doses that are not convulsant in control animals. According to these observations, it has been hypothesised that when alcohol intake is cut off, an enhanced NMDAR mediated neurotransmission underlies the observed neuronal hyperactivity [67, 69].

Indeed, several papers reported that chronic ethanol exposure leads to a selective enhancement of NMDAR function in cultured hippocampal [204, 173] and cortical neurons [31, 83, 148, 191]. For instance, while the amount of non-viable cells in hippocampal brain slice explants was significantly reduced in the presence of ethanol, cytotoxic effect of NMDA was significantly higher in ethanol-exposed samples after 24h withdrawal. Correspondingly, when cultures of rat cortical cells were treated with ethanol, the morphology of neurons was not altered, whereas obvious signs of neuronal damage and increased release of lactate dehydrogenase (LDH) were observed after 24 hours of withdrawal [148]. Interestingly, neurotoxic effect of ethanol withdrawal was observed only in those cultures, which were pre-treated with ethanol repeatedly, once daily at least for three consecutive days (Fig. **3A**) [149]. Furthermore, alcohol-withdrawal induced LDH-release was not observed when ethanol was continuously present (Fig. **3B**). In addition, whereas the effect of the $GABA_A$ receptor agonist muscimol was insignificant, NMDAR antagonists (MK-801 and ifenprodil) effectively reduced the neurotoxic effect of withdrawal [149]. Similarly, NMDA responses were found to be increased in cortical cultures treated with ethanol repeatedly for 3 days (Fig. **4**) [149, 150]. The 3-day repeated ethanol exposure paradigm used in these experiments is similar to the *in vitro* neuronal model described by Hu and Ticku [81] in which chronic but intermittent ethanol treatment (CIE) was used (12h ethanol followed by 12h withdrawal). The CIE exposure also produced enhanced NMDA mediated increase in intracellular calcium levels showing increased NMDA receptor functions. These data are consistent with the previous observations that acute administration of ethanol has a small neuroprotective effect and following long-term ethanol exposure and withdrawal neurons became more sensitive to NMDA [75, 173, 204]. These observations suggest that neuronal cells pre-treated with ethanol required further ethanol for survival i.e. became dependent on ethanol. These observations support the conception that NMDARs may play a crucial role in the development of *in vitro* ethanol dependence and alcohol-withdrawal evoked neurotoxicity. This *in vitro* test system, when ethanol treatment is interrupted and the cycle of treatment and withdrawal is repeated several times, can be used as an *in vitro* model for studying the development of ethanol dependence and withdrawal symptoms.

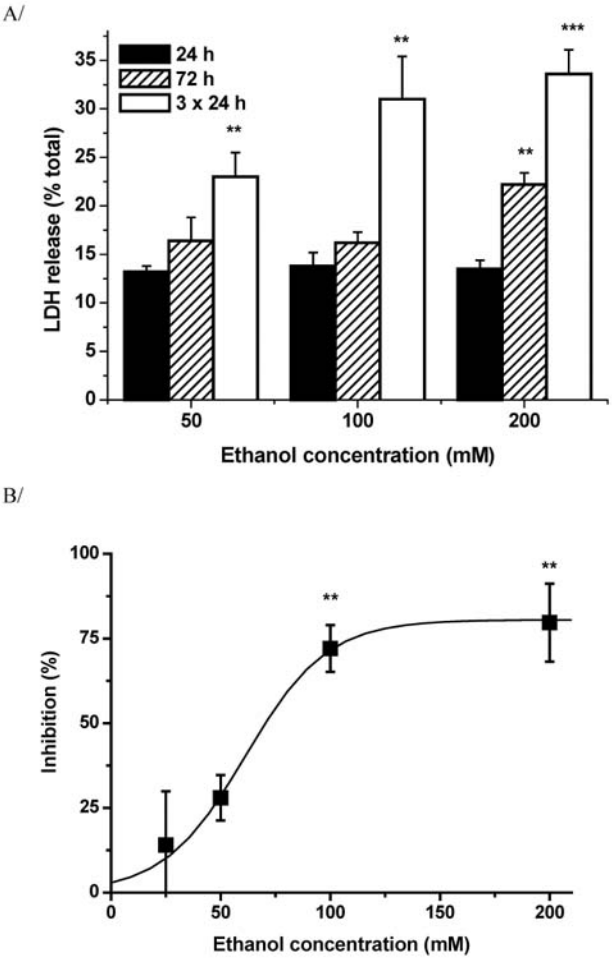

Fig. (3). Toxic effect of 24 h ethanol-withdrawal and its inhibition by re-addition of ethanol in ethanol pre-treated primary cortical cultures.

A) LDH activity of the culture medium expressed as percentage of total activity was measured in cultures pre-treated with different concentrations of ethanol once for 24 or 72 hours as well as daily for 3 successive days.

B) Inhibition of alcohol-withdrawal induced cytotoxicity by re-addition of ethanol in primary cultures of rat cortical neurones pre-treated with 100 mM ethanol daily for 3 successive days.

(*: p<0.05, **: p<0.01, ***: p<0.001 as compared to the respective control not treated with ethanol)

From: Nagy J., László L. (2002) Increased sensitivity to NMDA is involved in alcohol-withdrawal induced cytotoxicity observed in primary cultures of cortical neurones chronically pre-treated with ethanol. *Neurochem. Int.,* **40**, 585–591.

The CIE treatment is a widely used experimental paradigm also in animal studies and is a validated model for human alcohol withdrawal syndrome. In this kind of experiments rats are exposed to intermittent episodes of intoxicating doses of ethanol and withdrawal leading to a kindling-like state of behavioural excitability. It was observed that after repeated ethanol withdrawal experience

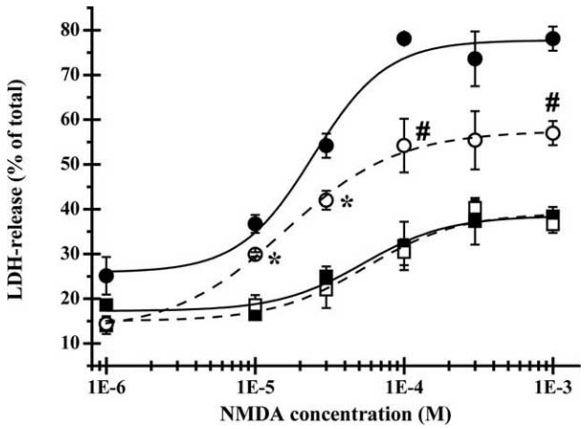

Fig. (4). Altered excitotoxic effect of NMDA after ethanol pre-treatment.

Effect of acute and chronic ethanol treatment on NMDA induced cytotoxicity. Control cortical cultures (squares) and cortical cultures pre-treated with 100mM ethanol repeatedly, once daily for 3 days (circles) were exposed to 300 μM NMDA for 15 min in the presence (open symbols) or absence (closed symbols) of 100 mM ethanol. LDH-release, expressed as percentage of total LDH content, was measured 24 h after NMDA wash out.

(*, P<0.01 compared to NMDA induced LDH-release in control cultures; #, P<0.01 compared to LDH-release in absence of ethanol).

reduced $GABA_A$ receptor function and increased NMDA receptor activity become exaggerated and these changes are suggested to have a role in the development of alcohol dependence, i.e. in manifestation of hyperexcitability when alcohol is withdrawn [10, 25, 138].

Effect of Long-Term Ethanol Exposure on the Structure of NMDARs

According to earlier reports, it was observed in both *in vivo* and *in vitro* studies that chronic ethanol treatment leads to an increase in the density of NMDARs leading to facilitated receptor functions [62, 64, 88, 89, 90, 147, 215]. However, up-regulation of NMDAR expression has not been found in all studies after chronic ethanol exposition [55, 186]. Consistent with the recently emerging view, the increased NMDAR function is presumably due to a differential up-regulation of the various NMDAR subunits. This conception is supported by several papers presenting evidence for altered NMDAR subunit composition after chronic ethanol treatment.

Notwithstanding, there is a disagreement in respect of the expression of the different NR subunits and NR1 splice variant forms. On one hand, some authors reported no changes in subunit expression at all [30], and others found changes solely in the expression of the NR1 [60] or NR2A [46] subunit in consequence of long-term ethanol exposure. On the other hand, there are papers concluding that besides several other types of subunits and certain NR1 splice variants, the expression of the NR2B subunit is increased. Lots of *in vitro* studies showed increased NR2B subunit

mRNA levels, with no change in NR1 and/or NR2A subunit transcription in cultured cortical neurons following chronic ethanol administration [73, 89, 143]. On the contrary, the levels of NR2B as well as NR2A and NR1 subunit proteins were found increased in the cortex and hippocampus of rats or mice [61, 62, 79, 96, 154, 207]. Furthermore, in cultured cerebellar granule cells, the 'developmental switch' of the NR2B subunit for NR2A was found delayed resulting in higher NR2B and lower NR2A subunit levels [194].

Similarly to these later observations, in primary cultures of cortical as well as hippocampal neurons from rats, the maximal inhibitory effect of ethanol as well as some NR2B subunit selective NMDAR antagonists on NMDA evoked cytosolic calcium elevations was significantly increased after ethanol pre-treatment [150]. However, the efficiency of the non-subunit selective NMDAR antagonist channel blocker MK-801 and the glycine site specific 5,7-DCK was not changed. Accordingly, increased expression of the NR2B subunits could be detected applying a flow cytometry based immunocytochemical method. Whereas, *in situ* immunocytochemical detection of the NR2B subunits could generate only qualitative data, the combination of immunocytochemistry with flow cytometry made an opportunity for a quantitative analysis of the expression. This quantitative analysis showed that the NR2B specific immuno-labelling was increased in a subpopulation of the cells in ethanol pre-treated compared to control cultures. According to similar analysis, the expression of the panNR1, NR2A, NR2C, and NR2D subunits was not changed after ethanol pre-treatment in rat cortical or hippocampal cultures (Fig. **5A**). In further studies, when the expression of the NR1 splice variants was investigated, similarly to the NR2B subunit, the expression of the C1 and C2' cassette containing splice variants was found to be increased in ethanol pre-treated hippocampal cultures (Fig. **5B**) [150].

Correspondingly, *in vivo* studies on rats also showed that after chronic ethanol ingestion the NMDA receptor function was enhanced in the lateral/basolateral amygdala. The increase in the NMDA receptor current density was associated with an increase in ifenprodil inhibition and a decrease in apparent calcium-dependent current inactivation. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) measurements demonstrated that the NR1 subunit mRNA expression, but not the NR2 or NR3 subunit transcription, was enhanced [60, 214].

The molecular mechanisms underlying these changes in subunit expression is one of the main questions in the near future. First results concerning the regulation of subunit composition by Ravindran and Ticku showed that the methylation status of the NR2B gene is altered following chronic ethanol treatment in mouse cortical neurons [177]. They found that demethylation this gene could be responsible for up-regulation of the NR2B subunit expression.

Consequences of Changes in Structure of NMDARs

The increased expression of the NR2B subunits accompanying with elevated levels of the C1 and C2' cassette containing splice variant forms of the NR1 subunits may underlie the enhanced NMDAR function. This idea is

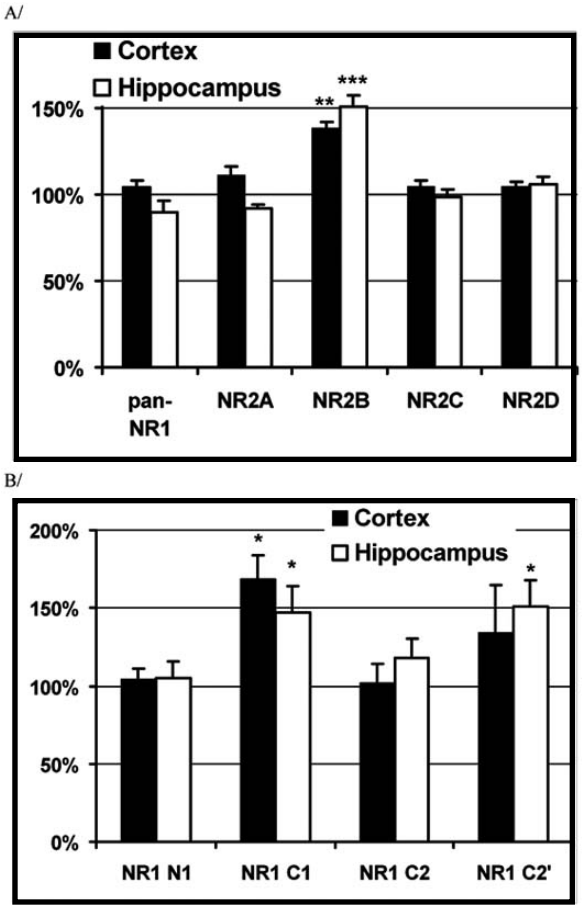

Fig. (5). Effect of chronic ethanol pre-treatment on the expression of different NMDA receptor subunits and NR1 splice cassettes.

Primary cortical and hippocampal cultures were treated with 100 mM ethanol daily for 3 days. Fixed samples were incubated in the presence of different NR2 and NR1 splice variant specific primary antibodies (Novus Biologicals). The binding of the primary antibodies was visualised *via* FITC-conjugated secondary antibodies (Sigma). Intensity of the subunit specific fluorescent labelling was analysed using a FACScan flow cytometer. The arithmetic mean FITC-fluorescence intensities were calculated from fluorescence histograms. The mean fluorescence values from samples incubated with the given NMDA receptor subunit specific antibody (NR staining) were corrected with the fluorescence of samples stained with an isotype specific control antibody (background). Each column represents the percentage of corrected fluorescence values obtained from ethanol pre-treated *vs*. control cultures.

(Each value represents mean + S.E. (bars); *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ compared to the control, paired t-test).

Data from: Nagy, J., Kolok, S., Dezső, P., Boros, A. Szombathelyi, Z. (2003) Differential alterations in the expression of NMDA receptor subunits following chronic ethanol treatment in primary cultures of rat cortical and hippocampal neurones. *Neurochem. Int.,* **42**(1), 35-43.

supported by the following observations: i) the deactivation time of NMDARs composed of NR1/NR2B subunits is longer than those built up of NR1/NR2A subunits [140], ii) the deactivation rate is four times faster for receptors composed of NR1 subunit containing the N1 cassette than those lacking this cassette [40, 187], and iii) NMDARs assembled of NR1 splice variants containing C1 and/or C2 cassettes may form functionally more active ion channels [175].

The phosphorylation states of the NMDARs are also altered after long-term ethanol exposure. It is known that Src family of protein tyrosine kinases, specifically c-Src and Fyn kinases potentiate the NMDA-activated currents in *in vitro* recombinant systems [8, 32, 101, 188] as well as in spinal neurons [211]. The up-regulation of NMDAR function by Src and Fyn accompanies with reduced sensitivity of NMDARs to ethanol [188]. Furthermore, phosphorylation status of NR1 and NR2B subunits was increased in the hippocampus of ethanol treated control but not in Fyn-deficient mice [95, 137]. This observation is in good agreement with that of Yaka *et al*. [221, 222], who found that the scaffolding protein RACK1 that binds Fyn kinase to the NR2B subunit dissociates from the complex due to ethanol exposure, consequently facilitating Fyn-mediated phosphorylation of the NR2B subunit leading to enhanced channel activity counteracting the inhibitory actions of ethanol. Reduced phosphorylation state of NR2 subunits – achieved by knocking out the Fyn kinase gene – increases ethanol sensitivity of NMDARs [7, 137]. In addition, transgenic mice over-expressing the Fyn tyrosine kinase and withdrawn from alcohol failed to show any increase of anxiety-like behaviour or reduction of exploratory activity like it was observed in case of their wild-type littermates [201]. This apparent lack of alcohol withdrawal-induced behavioural effects was associated with increased Fyn kinase activity and tyrosine phosphorylation of several proteins including the NR2B subunit.

Concerning the NR1 subunit, truncation ($NR1_{858stop}$) [7] or phosphorylation of Ser897 of this subunit decreased the ability of ethanol to inhibit NMDAR function. In addition, the reduced sensitivity of NMDARs to ethanol was linked up with the dopamine D1 receptor activation *via* dopamine and cAMP-regulated phosphoprotein-32 kD (DARPP-32) phosphorylation [126]. Activation of D1 receptors prevents the dephosphorylation of the NR1 subunit *via* a cascade that involves phosphorylation of PKA, which in turn phosphorylates dopamine and cAMP-regulated phosphoprotein-32 kDa (DARPP-32), which then inhibits the activation of PP1 phosphatase acting on the NR1 subunit [195]. *Via* this cascade, D1 receptor promotion of drug reinforcement, as might arise from prior exposure to drugs of abuse, reduces the sensitivity of NMDARs to blockade by ethanol [126] and may increase the motivational effects of ethanol [179].

Not only are the subunit composition and phosphorylation states of the NMDARs altered after long-term ethanol exposure but the localization of certain subunits. According to Carpenter-Hyland *et al*. [27], the co-localization of NR1 clusters with the presynaptic marker protein synapsin was increased in rat hippocampal neurons exposed to 50 mM ethanol for 4 days. This was accompanied by significant increases in the size and density of these synapsin-associated clusters with no change observed in non synapsin-associated NR1 clusters. Similar effects were observed with NR2B clustering after chronic ethanol exposure. The increase in synaptic NMDA receptor clustering was prevented by addition of a protein kinase A inhibitor or by co-exposure to a low concentration of NMDA and was reversed when ethanol was removed from the cultures. On the contrary, no changes were observed in the synaptic content, cluster size, or density of AMPA receptors after ethanol exposure. Electrophysiological measurements on ethanol-treated neurons revealed a similar enhancement in synaptic NMDA currents with no change in AMPA-mediated events.

Taken together, changes in subunit expression, phosphorylation states and synaptic clustering of NMDAR subunits due to long-term ethanol exposure may lead to the enhancement of NMDA responses. These changes may also explain the occurrence of acute ethanol tolerance leading to reinforcement of ethanol consumption and may underlie the development of physical dependence on ethanol and the increased sensitivity of neurons to excitotoxic insults.

Consequences of Increased NMDAR Function

Presumably in consequence of increased function of NMDARs, enhanced release of glutamate was observed after chronic ethanol exposure both in *in vitro* as well as *in vivo* experiments. Besides several other factors (e.g. functional deficits of GABA receptors and increased VGCC function [77, 212]), the NMDARs are major contributors to the increased glutamate release during alcohol withdrawal since in the brain of ethanol-dependent rats, the extracellular concentration of glutamate shows a transient, NMDAR mediated increase after cessation of ethanol intake and these changes are time-locked to the behavioural signs of ethanol withdrawal [44, 53, 183]. This enhanced glutamate release may contribute to the further shift towards the excitatory dominance in the CNS after ethanol withdrawal [184]. Furthermore, up-regulation of the NMDARs can enhance the activity of the noradrenergic system as well [51, 52], that may account for the vegetative instability seen in serious states of alcohol withdrawal, especially in delirium tremens [208, 209].

Increased calcium influx through NMDA receptors tightly coupled to calcium uptake into mitochondria causes the production of reactive oxygen species that interfere with the function of mitochondria. Primary inhibition of the mitochondrial respiratory chain can also indirectly induce further NMDA receptor stimulation. When the inhibitory action of ethanol on NMDA receptors is removed during withdrawal, the potential of neuronal injury is markedly increased through this system. Vulnerability of neurons is more pronounced when withdrawal kindling i.e. increased and/or prolonged withdrawal signs after repeated episodes of withdrawal, occurs [74].

Recently, it has been hypothesized that the same neuronal system including the mesolimbic dopaminergic pathway mediates the reinforcement for alcohol and other addictive drugs like opiates or cocaine [103, 158]. Indeed, ethanol has been reported to stimulate dopamine (DA) release in the

nucleus accumbens [103] and electrophysiological studies have demonstrated a concomitant ethanol-induced increase in the activity of ventral tegmental dopaminergic neurons [22, 23]. On the other hand, the observations that the NMDAR antagonist MK-801 increased burst firing of dopaminergic neurons [224] and could stimulate the release of DA from dopaminergic terminal areas suggest that glutamate - acting through NMDARs - exerts a tonic inhibitory action on DA release in the nucleus accumbens [83, 109]. According to the model of Fadda and Rossetti [53], blockade of the NMDARs by acute ethanol treatment disinhibits dopaminergic neurons *via* GABAergic interneurons possessing NMDARs. Withdrawal of alcohol, similarly to withdrawal of opiates or cocaine, has been found associated with decreased DA release in the limbic forebrain areas [182] due to decreased firing rate of dopaminergic neurons [49]. Thus, reduced dopaminergic functions seen after ethanol withdrawal may arise because of enhanced NMDA responses induced by chronic ethanol exposure.

All the above-discussed findings suggest the possibility that increased NMDA mediated neurotransmission may constitute the basis of both the motor signs (e.g. tremor, seizures etc.) and the affective or emotional disturbances (e.g. craving, dysphoria) associated with alcohol withdrawal. Bearing in mind that besides the glutamatergic system other transmitter mechanisms are also involved in the adaptive changes induced by chronic ethanol treatment and that *in vitro* experiments allow only limited conclusions to deduce for a whole organism, it is clear that alterations in NMDAR function may play a critical role in these processes leading to the development of alcohol dependence and withdrawal symptoms. According to this view of the pathomechanism of alcohol withdrawal syndrome, the NMDAR may be a possible target and NMDAR antagonists may be useful agents for the treatment of both the physical and the psychical signs of alcohol withdrawal.

NMDAR ANTAGONISTS IN PHARMACOTHERAPY FOR ALCOHOL WITHDRAWAL

Current pharmacotherapies for alcohol dependence, disulfiram and naltrexone, aiming at alleviating symptoms of acute abstinence and minimising the risk of relapse show limited efficacy in large multicenter studies [65, 111]. Also, agents, which appear to target the glutamatergic system, are emerging as an additional therapeutic option [70, 85, 87, 110, 129, 130, 218]. In the early 90s, it was already hypothesized that NMDAR antagonists can block alcohol withdrawal induced seizures in ethanol dependent animals. Since then, extensive literature on animal experimental and preliminary clinical data suggest that NMDAR antagonists are promising candidates for the treatment of alcohol withdrawal symptoms, inasmuch as these compounds may attenuate not only the physical but also the affective and motivational components of AWS. However, the field of alcoholism research is in the relatively early phases of determining the extent to which glutamatergic agents might reduce alcohol consumption and relapse.

Acamprosate

The American Food and Drug Administration (FDA) recently granted the approval of a novel anti-alcohol medication Campral (acamprosate calcium) to maintain abstinence in patients with alcohol dependence. In Europe, more than 4 million people have been treated with this agent since 1989, when it became commercially available in France. According to several human studies, acamprosate has a consistent effect on prolonging abstinence and reducing the rate of relapse, in conjunction with an equally consistent absence of effect on self-reported craving, suggesting that it can be used as a relapse-prevention medication. In previous animal studies, it was observed that acamprosate dose-dependently reduced alcohol consumption and hypermotility during ethanol withdrawal with no effects on food and water intake and without any effects generalize to those of ethanol. In addition, acamprosate did not substantially alter the discriminative stimulus properties of ethanol, pentobarbital, or amphetamine [196, 197, 198].

The exact mechanism of action of acamprosate, originally developed as a GABA analogue, was intensively investigated in the past years (for a review see [217]). Since acamprosate is chemically similar to GABA, early studies indicated that acamprosate interacts with the GABAergic system [16] to affect behaviours related to ethanol consumption. However, this interaction of acamprosate with the GABA receptors does not appear to be comparable to the effects induced by either benzodiazepines or barbiturates since acamprosate cannot be substituted for GABA agonists in a drug-discrimination procedure [68]. In addition, it would not bind to recombinant or native $GABA_A$ receptors in transfected HEK 293 cells or enhance chloride currents in these receptors [226]. The observation of Dachour *et al*. [41, 42, 43] i.e. that acamprosate can lessen the increase in extracellular glutamate level in microdialysates from nucleus accumbens during ethanol withdrawal was an important finding for the therapeutic use of this agent.

According to other studies it was suggested that acamprosate has an inhibitory effect on the native or recombinant NMDA-receptors as well as on voltage-sensitive Ca^{2+} channels [1, 176, 199]. As acamprosate reversed the potentiating effects of spermine, it was thought that acamprosate may act at the polyamine site of the NMDA receptor [171]. Indeed, acamprosate effectively reduced both the enhanced glutamate-induced calcium entry and neurotoxicity in ethanol pre-treated primary cultures of organotypic hippocampal [133] or neocortical neurons from rats in a concentration-dependent manner (Fig. **6A**) [4, 153]. However, its protective effects against glutamate-induced neurotoxicity were observed only in ethanol-withdrawn cultures. Furthermore, although acamprosate significantly reduced the calcium entry caused by glutamate or K^+ in control and ethanol-exposed cultures, the neuroprotective effects of the drug did not correlate with its effects on calcium entry, making it unlikely that acamprosate directly affects NMDA receptors *via* the glutamate binding site or the receptor-operated calcium channel [4]. This idea was confirmed by further studies which found that acamprosate has no direct effect on the NMDARs [4, 132, 153, 171, 196]. Al Qatari *et al*. [3] argued that acamprosate may have an excitatory or inhibitory effect on NMDA receptors depending upon the experimental conditions indicating that acamprosate, at least partly, acts as a `partial agonist' at the NMDA receptor.

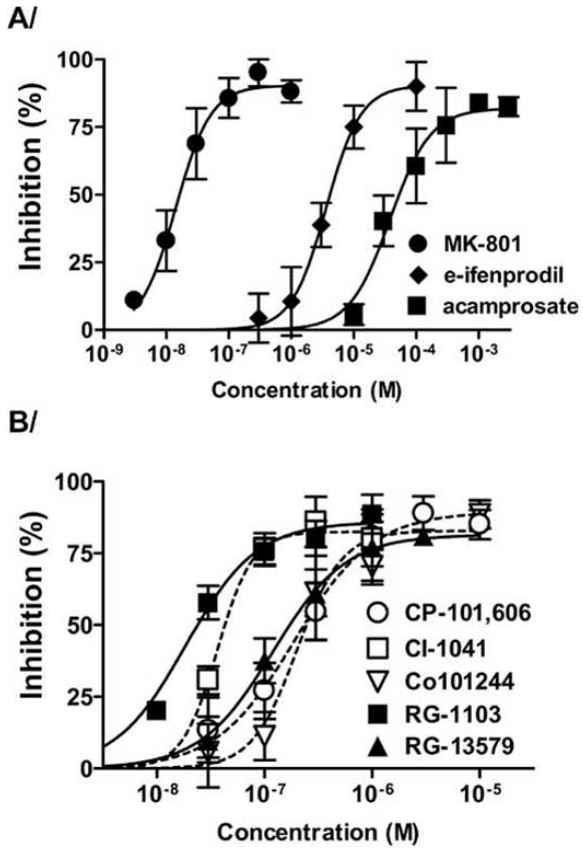

Fig. (6). Inhibitory effect of NMDAR antagonists on ethanol-withdrawal-induced neurotoxicity.

Neuronal cell death caused by 24-hour ethanol-withdrawal in primary cultures of rat cortical neurones pre-treated with 100 mM ethanol for 3 consecutive days was quantified by measuring LDH-release. Different concentrations of MK-801, erythro-ifenprodil and acamprosate (panel A) or some known (open symbols, dashed lines) and novel (filled symbols, straight lines) NR2B SSNAs (panel B) were present during the withdrawal period. Each point represents the percentage of inhibition (mean ± S.E. (error bars)).

From: Nagy, J., Horváth, C., Farkas, S., Kolok, S., Szombathelyi, Z. (2004) NR2B subunit selective NMDA antagonists inhibit neurotoxic effect of alcohol-withdrawal in primary cultures of rat cortical neurones. *Neurochem. Int.,* **44**(1), 17-23.

According to the observations of Harris *et al*. acamprosate displaced [^{3}H]glutamate but did not compete with NMDA for [^{3}H]glutamate binding sites in membrane preparations of cortices, cerebellums, and hippocampi of rats. Furthermore acamprosate displayed total competition with trans-ACPD (1-aminocyclopentane-trans-1,3-dicarboxylic acid) an agonist at both group I and group II metabotropic glutamate receptors and similarly to SIB-1893, a non-competitive antagonist at the mGluR5 receptor, it was neuroprotective against trans-ACPD induced neurotoxicity that likely results from mGluR mediated potentiation of NMDARs [76]. Also, in the CA1 region of ethanol pre-treated organotypic hippocampal slices, where neurotoxicity was observed after a 24-hr withdrawal, acamprosate, as well as SIB-1893, MK-801, and staurosporine were all neuroprotective. In this ethanol pre-treated slice culture preparations the polypeptide levels of mGluR5 receptors were found to be increased [77], similarly as the NR1 and NR2B subunits of NMDARs in other neuronal cultures after long-term ethanol exposure [150, 176]. Considering these observations, acamprosate may act on the mGluR5 receptors reducing its positive feedback control over the NMDARs [217]. Although the exact mechanism of action of acamprosate is still a matter of debate, the glutamatergic hypothesis may help to explain many of the effects of acamprosate in human alcohol dependence, especially in the acquisition of cue-elicited drinking behaviours [17, 41, 80, 86, 116, 117, 199].

Competitive and Channel Blocking NMDAR Antagonists

So far, the classic competitive and channel blocking NMDAR antagonists were tested and proved useful in *in vitro* or animal models of alcoholism. Early experiments showed that competitive NMDA receptor antagonists acting at the glutamate binding site (e.g. CGP 39551, D-CPP-ene) decreased handling-induced hyperactivity after withdrawal from chronic ethanol treatment in mice [113, 119, 178] and reduced alcohol deprivation effect (i.e. an overshoot in alcohol consumption shown by animals subjected to forced abstinence from regular drinking when ethanol is again available [105]) in rats [210]. These compounds increased the threshold for population spikes in hippocampal slices from the same animals. NMDAR antagonists acting within the ion channel (e.g. ketamine, MK-801 and ADCI) were also shown to suppress withdrawal-induced seizures effectively in both rats and mice [56, 71, 142]. Unfortunately, preclinical studies indicated that most of these compounds produce psychotomimetic or sedative effects, ataxia, muscle relaxation, neuronal damages in the cingulate cortex as well as motor and learning impairment. These serious side effects impeded their introduction to the human therapy [20, 28, 100, 145, 164, 223]. However, due to immense therapeutic promise of NMDA antagonists in acute and/or chronic neurodegenerative and psychiatric disorders efforts have been made to develop compounds lacking these side effects. More encouraging approaches were performed with low affinity channel blockers like memantine or with NMDA antagonists acting at the glycine binding site (L-701,324) having more tolerable side effect profiles.

Novel channel blockers like memantine (1-amino-3,5-dimethyl-adamantane) and its analogue neramexane (MRZ 2/579, 1-amino-1,3,3,5,5-pentamethyl-cyclohexane hydrochloride) have improved side effect profile probably due to their moderate potency and rapid, strongly voltage-dependent blocking kinetics [165]. These compounds greatly inhibited alcohol consumption without affecting water or food intake during relapse in long-term voluntarily alcohol-drinking rats [85, 87, 169, 190]. In addition, neramexane as well as memantine effectively suppressed ethanol withdrawal induced seizures in alcohol dependent rats [12]. According to recent results by Kotlinska *et al*. chronic administration of neramexane inhibits the development of ethanol dependence, reflected as a decrease in ethanol withdrawal-associated audiogenic seizures as well as the acquisition and expression of ethanol-induced place preference [108].

Glycine-B Site NMDAR Antagonists

Glycine-B site antagonists were also shown to attenuate the expression of alcohol withdrawal symptoms [45]. A member of this type of NMDAR antagonists, L-701,324 (7-chloro-4-hydroxy-3-3-phenoxyphenyl-2-1H–quinolone) produced a dose-dependent inhibition of audiogenic seizures associated with alcohol withdrawal [106, 107] and potently blocked the acquisition of ethanol-induced conditioned place preference [11] and reduced alcohol consumption during alcohol deprivation [210]. Preliminary problems with glycine site antagonists included poor systemic availability has now been overcome with agents like GV196771A ((E)-4,6-Dichloro-3-(2-oxo-1-phenylpyrrolidin-3-ylidenemethyl)-1H-indole-2-carboxylic acid sodium salt) or SM-31900 (3(S)-(2-(4- (Amino methyl) -2- (1(R)-carboxyethoxy)phenylamino) -2- oxoethyl) -7- chloro -1,3,4,5- tetrahydrobenzo(c,d)indole -2- carboxylic acid hydrochloride) which are not only of very high affinity, but also have improved pharmacokinetic and physicochemical properties, such as good brain permeation and solubility [94]. Although these compounds were not tested in animal models related to alcoholism, the facts that SM-31900 has a potent anticonvulsant activity [97], GV196771A can inhibit the development of morphine tolerance [174] and both compounds are devoid of behavioural side effects (hyperactivity, motor dysfunction) make these compounds promising candidates also for the treatment of alcoholism.

NR2B Subunit Selective NMDAR Antagonists

In recent years, novel non-competitive NMDAR antagonists inhibiting the NR2B subunit containing NMDARs have emerged and received considerable attention. Although, this type of compounds was initially thought to interact with the polyamine site, recent experiments using chimeric NR2A/NR2B subunits revealed that the major determinant of the inhibitory effect of ifenprodil – the initial chemical lead of the NR2B subunit selective antagonists – localizes to a distinct site of the NR2B subunit [66, 34]. The NR2B subunit selective antagonists showed potency in animal models of neurodegeneration [99], Parkinson disease [155, 156, 200, 220], and hyperalgesia [19, 29, 36, 57]. It was also realized that this type of compounds lacks the serious side effects of the classic NMDAR antagonists' [162]. Although, like other un-competitive NMDAR antagonists they may have some adverse effect on learning and memory, it was proved that they have a wider separation between doses that are effective in seizure or stroke models and those that disrupt learning and memory. The limited information on the novel NR2B subunit selective antagonists (Table **1**) such as CP-101,606 (traxoprodil) [33, 98], Ro25-6981 [59], Co-101244 [225], CI-1041 [35] and RG-1103 [18] also suggests that these drugs are better tolerated and are largely devoid of adverse CNS effects at antinociceptive doses, at least with respect to psychotomimetic, ataxic and sedative effects [19, 29, 35, 57, 146, 203].

Previously, ifenprodil and its analogue eliprodil were found effective in animal models of alcohol dependence. According to Malinowska *et al*. [128], ifenprodil potently reduced the severity of withdrawal-induced seizures. Oral administration of increasing doses of eliprodil produced a dose-dependent and almost complete inhibition of ethanol withdrawal produced audiogenic seizures in alcohol dependent Sprague-Dawley rats [106]. This effect of eliprodil was achieved at doses that by themselves did not alter the basal locomotor activity of untreated control animals. Similar results were observed with ifenprodil in

Table 1. Some NR2B Subunit Selective NMDA Antagonists

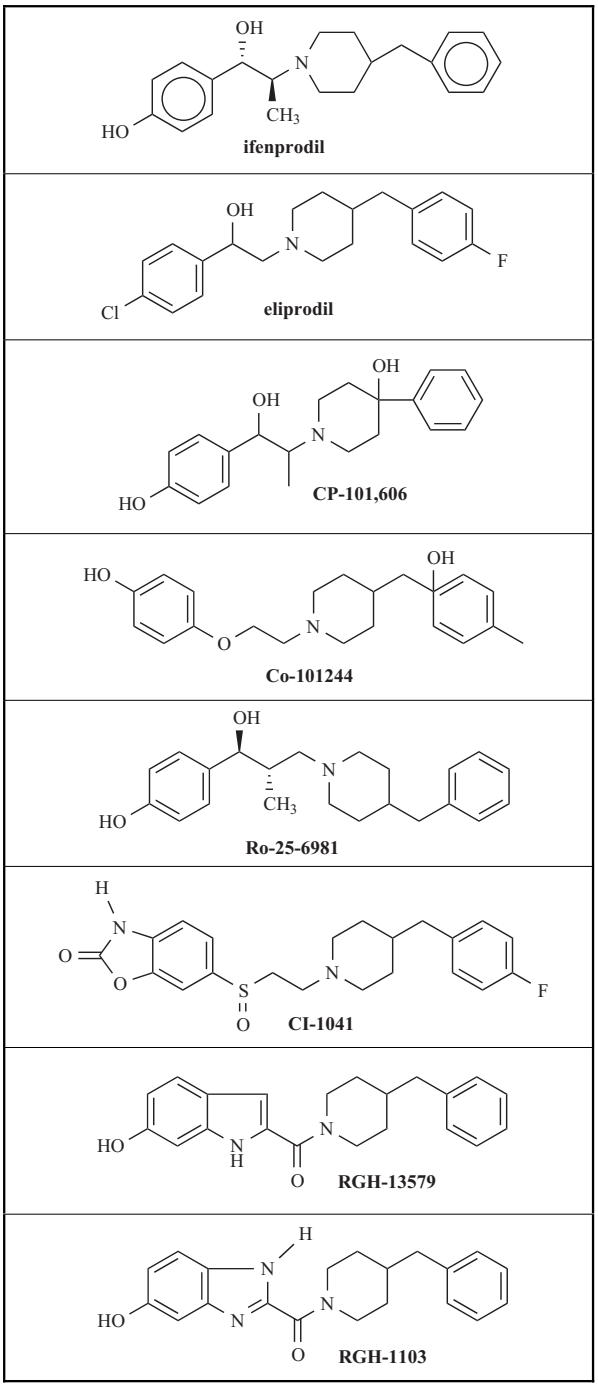

Table 2. Inhibitory Effect of NMDA Antagonists on Cytosolic Calcium Rises and Ethanol Withdrawal Induced Neurotoxicity

Compound	NMDA evoked Ca^{2+} elevation		withdrawal induced toxicity	
	I_{max} (%)	IC_{50} (μM)	I_{max} (%)	IC_{50} (μM)
MK-801	100 ± 5	0.037 ± 0.002	90 ± 5	0.020 ± 0.004
CP-101,606	58 ± 2	0.041 ± 0.005	89 ± 9	0.201 ± 0.061
Co-101244	67 ± 2	0.024 ± 0.003	101 ± 5	0.206 ± 0.045
CI-1041	70 ± 3	0.007 ± 0.001	82 ± 6	0.037 ± 0.008
RGH-13579	77 ± 3	0.018 ± 0.004	81 ± 18	0.137 ± 0.067
RGH-1103	74 ± 2	0.002 ± 0.0004	87 ± 6	0.019 ± 0.005
Acamprosate	–1.2 ± 2.9	> 300	82 ± 8	40 ± 13

Compounds were tested for their inhibitory effect on cytosolic calcium elevation evoked by 40 μM NMDA and on ethanol-withdrawal produced neurotoxicity in primary cultures of rat cortical neurons.

From: József Nagy, Csilla Horváth, Sándor Farkas, Sándor Kolok, Zsolt Szombathelyi. (2004) NR2B subunit selective NMDA antagonists inhibit neurotoxic effect of alcohol-withdrawal in primary cultures of rat cortical neurones. *Neurochem. Int.,* **44(1),** 17-23.

alcohol dependent male C57BL/6J mice [171]. The expression of spontaneous ethanol withdrawal signs (piloerection, jerk, tremor) occurring 6 – 12 hours after the discontinuation of ethanol treatment was suppressed by ifenprodil. According to Kotlinska and Liljequist [107], eliprodil effectively reversed the reduction in extracellular DA level during ethanol withdrawal but only partially and dose-independently substituted ethanol indicating that it has no discriminative stimulus properties similar to those produced by ethanol. Furthermore, ifenprodil dose-dependently reduced the expression of an alcohol deprivation effect as well [210].

With the novel NR2B subunit selective NMDAR antagonists so far only *in vitro* experiments were reported. In primary cultures of cortical neurons from rats pre-treated with ethanol intermittently for 3 days, CP-101,606, Co-101244 and CI-1041 as well as some of the novel indole-2-carboxamide derivative NR2B subunit selective antagonists (RGH-13579 and RGH-1103 [18]) potently and dose-dependently reduced the withdrawal-evoked LDH release (Fig. **6B**). One of the novel compounds (RGH-1103) was as effective as MK-801, the most potent but not subunit selective NMDAR antagonist. The inhibitory potencies of the NR2B subunit selective antagonists for withdrawal-induced toxicity was in good linear relation with their effectiveness for inhibition of NMDA induced cytosolic calcium elevation (Table **2**) [153].

SUMMARY

According to the recently emerged glutamatergic theory of the pathomechanism of alcohol withdrawal syndrome, increased NMDA receptor function may play a central role in the development of alcohol dependence and manifestation of the withdrawal symptoms. Despite the challenging complexity of ethanol's action, there is now a convergence of evidence to indicate that i) the capacity of ethanol to block NMDARs is an important component of the human behavioural and intoxicating effects of ethanol, ii) ethanol tolerance and dependence are associated with alterations in NMDAR function that promote heavy drinking by reducing the negative consequences of ethanol intoxication, iii) physical ethanol dependence is associated with upregulation of certain NMDAR subunits and iv) acute ethanol withdrawal is associated with increased glutamatergic activity [98]. Along with the long-term ethanol exposure evoked structural changes in NMDARs, an increased expression of the C1 and C2' cassette containing splice variant forms of the NR1 as well as the NR2B subunits contributes to the elevated function of these receptors [140, 151, 152]. Although, only few of the novel NMDAR antagonists have been examined in animal models of alcoholism, they were found effective with encouraging *in vitro* and – according to the available preliminary data, *in vivo* efficiency. Since "classic" NMDAR antagonists reduce hyperactivity, seizures, and neuronal cell loss as well as restore normal brain levels of glutamate and DA associated with ethanol withdrawal, NMDAR antagonists may have a role in the pharmacotherapy of withdrawal symptoms as well as in the prevention of relapse and maintenance of abstinence. To prove that the novel NMDAR antagonists, including the NR2B subunit selective ones are positively useful in the pharmacotherapy for alcoholism is a major challenge for the forthcoming years.

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