The NR2B Subtype of NMDA Receptor: A Potential Target for the Treatment of Alcohol Dependence

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Abstract: Ethanol is a small molecule acting on several neurotransmitter systems in the brain. Accumulating evidences suggest that the primary excitatory – i.e. the glutamatergic – neurotransmitter system is a particularly important site of ethanol's action. Several studies showed that ethanol is a potent and selective inhibitor of the N-methyl-D-aspartate (NMDA) receptors and prolonged ethanol exposition leads to a compensatory "up-regulation" of these receptors resulting in enhanced NMDA receptor-mediated functions after removal of ethanol. These alterations are supposed to contribute to the development of ethanol tolerance, dependence as well as the acute and delayed signs of ethanol withdrawal.

In recent papers, alterations in subunit composition of NMDA receptors were reported after long term ethanol exposure. mRNA and/or protein levels of NR2A and NR2B types of subunits were found elevated both by *in vivo* and *in vitro* experiments. Our results showed that especially the NR2B subunit expression is increased in cultured hippocampal and cortical neurones after 3 days of intermittent ethanol treatment. According to the high calcium permeability, the increased agonist sensitivity and the relatively slow closing kinetics of NMDA ion channels composed of NR2B subunits, the above mentioned changes may underlie the enhanced NMDA receptor activation observed after long term ethanol exposure. Accordingly, we have tested NR2B subunit selective NMDA receptor antagonists in primary cultures of rat cortical neurones pre-treated with ethanol intermittently for 3 days and found that these compounds potently inhibited the neurotoxic effect of ethanol withdrawal. Hypothesising the involvement of enhanced NR2B subunit expression in development of alcohol dependence and withdrawal symptoms and considering the tolerable side effect profile of the NR2B subunit selective NMDA receptor antagonists, the NR2B type of NMDA receptor subunit may serve as a possible drug target in pharmacological interventions for alcoholism.

The aim of this review is to give an update on the role of altered structure and function of NMDA receptors after ethanol exposure and to summarise the recent data about the activity of NR2B subunit selective NMDA receptor antagonists in model systems related to alcoholism.

Keywords: alcoholism, alcohol dependence, alcohol withdrawal, NMDA receptor, NR2B subunit, NR2B subunit selective antagonist.

INTRODUCTION

Alcohol dependence is a source of major public health, social and medical problems all over the world. Ethanol interferes differentially with the neurotransmission processes in the CNS affecting many of the known transmitter systems. The brain's major inhibitory (gamma-aminobutyric acid (GABA)) and excitatory (gultamate) amino acid transmitter systems have been shown to be involved in the mediation of the behavioural, neurophysiological and pathological effects of ethanol [1-4]. In the past years there has been increasing evidence that acute ethanol facilitates GABAergic transmission (by enhancing chloride conductance through the $GABA_A$ receptor) and inhibits glutamatergic function (by decreasing cationic conductance). In this later case, especially the N-methyl-D-aspartate type of glutamate receptors (NMDARs) seems to be an important site of ethanol's action. Acutely, alcohol exerts an antagonistic effect on NMDAR function [5-7], and – presumably due to compensatory changes – chronic alcohol consumption leads to an increase in NMDAR-mediated neurotransmission [8-11]. This latter alteration is presumably not due to an overall increase in the NMDA receptor density, but to differential up-regulation of the various NMDAR subunits resulting in alterations in the composition and consequently in the functional properties of NMDAR complexes [12-15]. Therefore, modulators of the glutamatergic/NMDAR system are now believed to be useful pharmacotherapeutic agents in the treatment for alcohol dependence [16].

STRUCTURE AND FUNCTION OF NMDA RECEPTORS

NMDARs are members of ionotropic glutamate receptors and are highly expressed in the CNS. These receptors have central roles in excitatory synaptic transmission, in synaptic plasticity and excitotoxicity. The involvement of NMDARs in these diverse processes rest on their unique features, which include voltage-sensitive block by extracellular Mg^{2+},

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a high permeability to Ca^{2+} and unusually slow 'activation/deactivation' kinetics. The main agonist of NMDARs, glutamate, can open the ion-channel permeable for Na^+ and/or Ca^{2+} only if the plasma membrane became depolarised and so the Mg^{2+} blockade was displaced. So, NMDARs act as coincidence perceptive elements, which became active only when electrical and chemical signals are present concurrently. NMDARs also display sensitivity to several other endogenous modulators. For full receptor activation, another ligand, the co-agonist glycine must also bind to the receptors. Extracellular Zn^{2+} and polyamines also modify the behaviour of NMDARs, whereas physiological levels of protons suppress NMDAR activation. In addition, NMDARs interact with various intracellular scaffolding, anchoring and signalling molecules associated with the postsynaptic density. 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 [17, 18] (Fig. (1)).

Several distinct NMDAR subtypes have now been identified in neurones. The ubiquitously expressed NR1 subunit occurs as eight distinct isoforms as a consequence of three independent sites of alternative splicing. Families of four distinct NR2 (A, B, C and D) and two NR3 (A, B) subunits are also identified. Although, the precise subunit composition and stoichiometry of native NMDARs is still a matter of debate [19, 20], NMDARs are thought to exist as tetrameric complexes consisting of at least one NR1 and one NR2 subunit [21-23]. The subunits are most probably arranged as dimer of dimers in the receptors with an NR1-NR1-NR2-NR2 orientation in the channel [24]. Each subunit has four hydrophobic regions, although only three form membrane spanning domains (TM1, TM3, and TM4). The fourth one (M2) makes a hairpin bend within the membrane and participate in the formation of the channel pore [17].

The major subunit dependent properties of NMDARs include their single-channel conductance [25] and their block by extracellular Mg^{2+}. Diheteromeric NMDARs containing NR1/NR2A or NR1/NR2B subunits generate 'high-
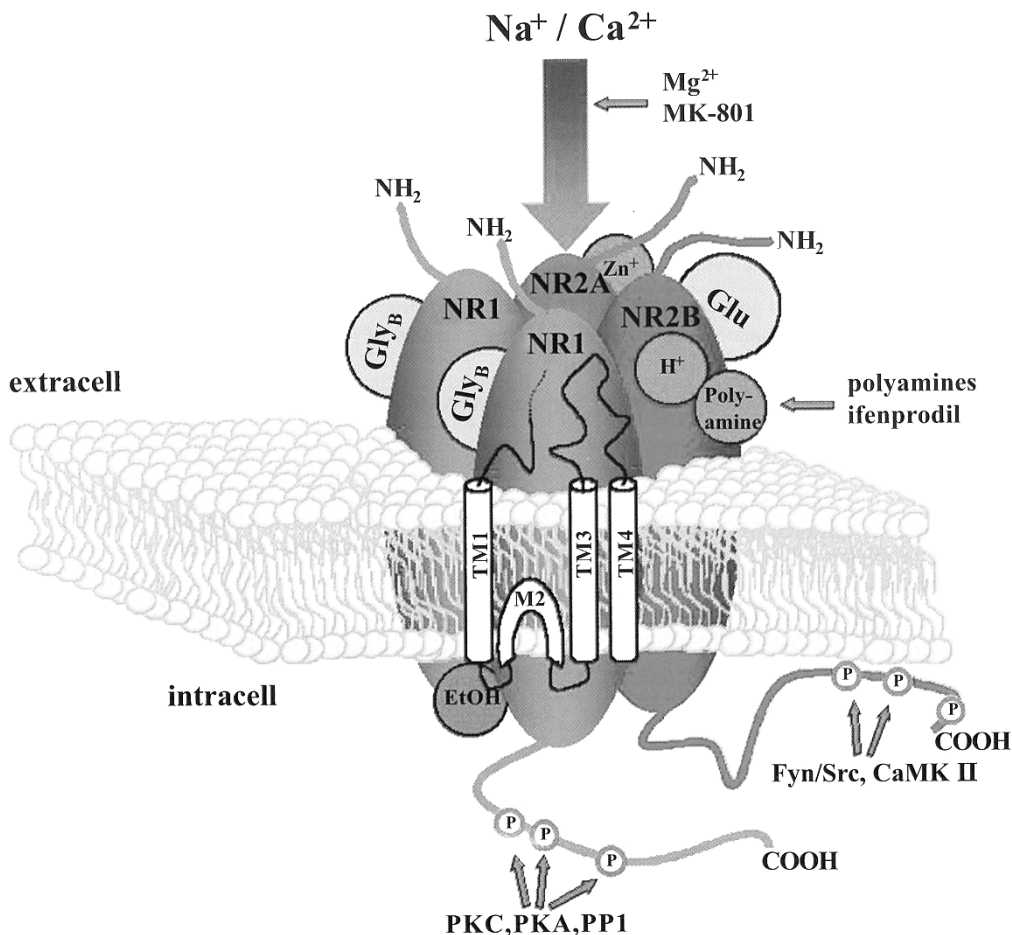

Fig. (1). Schematic model of the NMDA receptor

Model showing the heteromeric assemblies of four subunits in NMDA receptors. Each subunit has four hydrophobic regions, although only three form membrane-spanning domains (TM1, TM3, and TM4). M2 makes a hairpin bend within the membrane and forms the channel pore. Functional NMDA receptor complexes are formed by combinations of NR1 and NR2 subunits, which contain the glycine and glutamate recognition sites, respectively. Supposed binding sites of agonists, some antagonists and other modulators are shown.

conductance' channel openings with a high sensitivity to Mg^{2+} blockade. On the contrary, receptors containing NR2C or NR2D subunits give rise to 'low-conductance' openings with a lower sensitivity to extracellular Mg^{2+}. The NR3 subunits that are thought to be regulatory in nature, since they do not form functional channels alone but can co-assemble with NR1/NR2 complexes [26] can also give rise to low-conductance channel openings. When NR3 subunits are co-assembled with NR2A these channels show a roughly fivefold reduction in relative Ca^{2+} permeability as compared with NR1/NR2A assemblies [27].

The NR2B subunit appears to be important in respect of several functional properties of the NMDAR. It participates in regulation of the Mg^{2+} block, since replacement of Trp607 to Asp or Ala greatly attenuates or to Leu completely abolishes the Mg^{2+} block. Williams *et al.* [28] thus proposed a model in which the M2 loop of NR2B holding the Trp607 is part of the binding site for Mg^{2+}. The NR2B subunit affects the binding of glycine as well. Whereas the binding site for glycine is thought to be located on the NR1 subunit [17], a high-affinity site for CGP61594, a glycine site antagonist, was shown to be exclusively displayed by NR1/NR2B receptors as compared with receptors composed of other types of NR2 subunits [29]. The NR2B subunit is highly relevant for mediating modulatory effects of polyamines, too. Polyamines, such as spermine and spermidine, appear to facilitate NMDAR function, probably by reducing the inhibitory impact of protons [30]. In addition, similarly to polyamines, ifenprodil, the prototype of the NR2B subunit selective NMDAR antagonists (SSNAs) and related compounds – e.g. eliprodil, CP-101,606, Ro25-6981 [31-34] – antagonise NMDA responses with a preference for the NR2B subunit [35]. Binding assays performed in HEK-293 cells expressing recombinant NR1/NR2B or NR1/NR2A/NR2B receptors suggest two classes of NR2B SSNAs. Whereas antagonists like Ro25-6981 binds NR2B-containing receptors regardless of the NR2 subunit composition, the binding of antagonist such as CP-101, 606 is affected significantly by the presence of another type of NR2 subunit within the receptor complex [36].

EFFECT OF ETHANOL ON NMDA RECEPTOR FUNCTION

Acute Effects of Ethanol

Glutamatergic neurotransmission is considered as one of the main components of the pathway involved in the mediation of the addictive effect of alcohol [37]. Besides AMPA receptors, especially the NMDA type of glutamate receptors belongs into the highest affinity targets for ethanol in the CNS [38, 39]. Biochemical, electrophysiological and behavioural evidences show that ethanol is a potent and selective inhibitor of these receptors [40, 41].

Several studies involving recombinant receptors have demonstrated that receptors composed of different NR2 subunits have differential sensitivity to the inhibitory effect of ethanol. The ability of ethanol to depress current responses to NMDA paralleled with the action of ifenprodil in rat cultured cortical neurones [42]. Similar results were obtained when the NMDA-induced release of

[^{3}H]noradrenaline was measured in slices of the rat cerebral cortex [43]. Since ifenprodil is known as an NR2B selective antagonist [31], it was assumed that ethanol acts on the NR2B subunit. Indeed, studies performed on recombinant NMDARs showed that heteromers containing either the NR2A or NR2B subunits are preferentially sensitive to ethanol inhibition vs. heteromers containing the NR2C or NR2D subunits [44-49]. Moreover, NMDARs with NR1/NR2B subunit combination were more susceptible to the effect of ethanol than those composed of NR1/NR2A subunits [51-53].

Earlier it was thought that ethanol bound to a hydrophobic pocket distinct from other modulatory binding sites of the NMDAR [54,55]. Recently it was suggested that this pocket is associated with the third transmembrane domain (TM3) of the NR1 subunit. 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, substitution of the slightly larger Trp residue at Phe639 resulted in receptors that were slightly more sensitive to ethanol inhibition than wild-type receptors. These observations suggest that some physical or chemical properties of the 639 position of the NR1 subunit may be an important determinant of ethanol sensitivity [56, 57]. There is another possibility, namely that the action of ethanol on the NMDAR is mediated by changes in the phosphorylation status of the receptor subunits. According to Alvestad *et al.* [58] ethanol has an effect on tyrosine phosphorylation of both NR2A and NR2B subunits. Phosphorylation states of tyrosine residues in these subunits were significantly reduced following *in situ* exposure of hippocampal slices to 100 mM ethanol. Especially, the phosphorylation of Tyr1472 on NR2B subunit was reduced. According to other authors, ethanol administration may increase the phosphorylation of the NR2B [59] or NR2A [52] subunits by Fyn kinase, a member of the Src family of tyrosine kinases. It is thought that this change reduces NMDAR sensitivity to ethanol leading to development of tolerance. Although these data are equivocal, these observations suggest a possible indirect mechanism by which ethanol may modulate NMDAR functions via tyrosine kinases and/or phosphatases [60, 61].

Chronic Effects of Ethanol

Data from studies on neuroadaptation following chronic exposure to ethanol indicate a significant role of increased NMDAR function in expression of alcohol withdrawal syndrome as well as in associated neuronal damage. Initial *in vivo* studies showed that seizures evoked by withdrawal of ethanol were exacerbated by administration of NMDA at doses that are not convulsant in control animals, and attenuated by NMDAR antagonists. According to these data, it has been hypothesised that the increase in NMDAR mediated neurotransmission following the removal of ethanol blockade mediates ethanol withdrawal seizures [62, 63]. Indeed, chronic ethanol exposure leaded to a selective enhancement of N-methyl-D-aspartate receptor function in cultured hippocampal and cortical neurones [10, 11,64-66]. Similarly, in hippocampal [67, 68] and cortical [69] slices prepared from mice chronically treated with ethanol, electrical stimulation evoked synaptic potentials, which
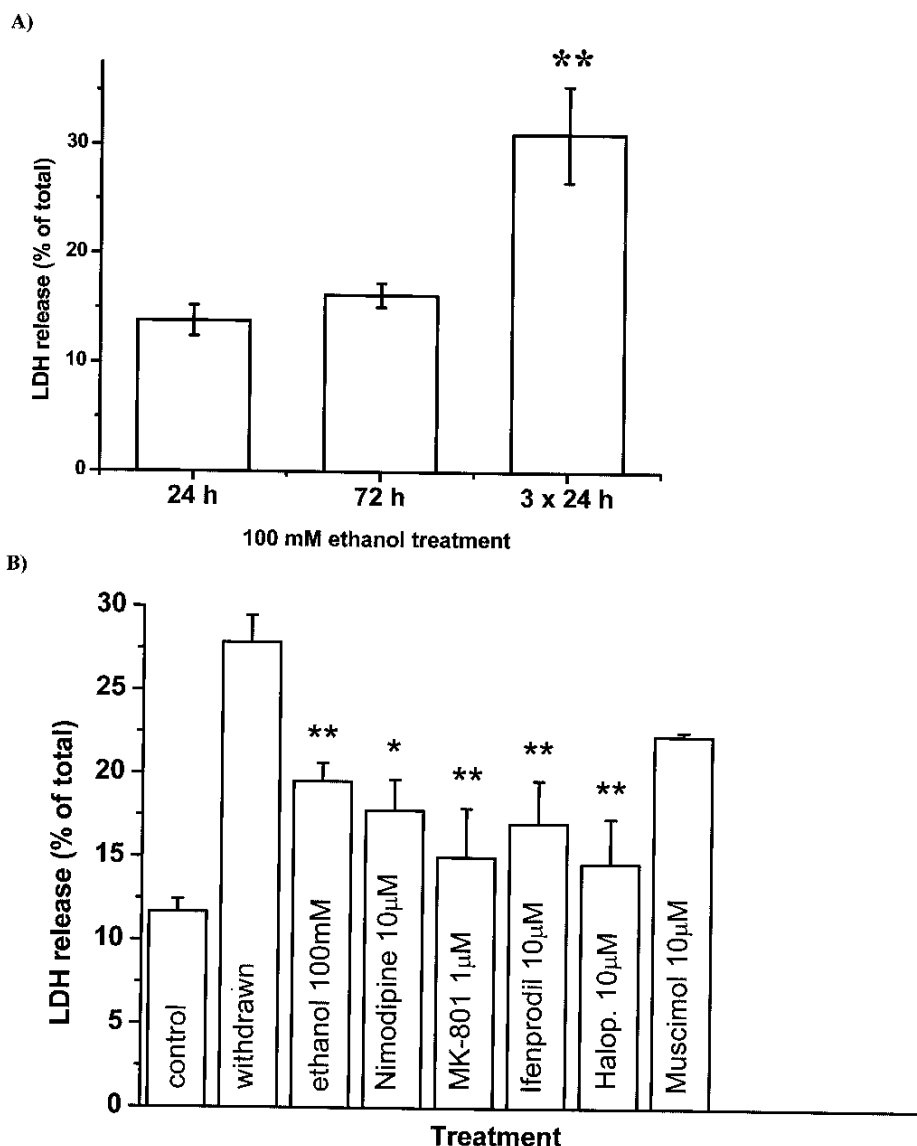
A)

100 mM ethanol treatment

B)

Treatment

Fig. (2). *In vitro* model of ethanol dependence

A. Toxic effect of ethanol-withdrawal

Released LDH activity in the culture medium after 24 h withdrawal was measured in primary cultures of rat cortical neurones pre-treated with 100 mM ethanol once for 24h, 72h or daily for 3 successive days. Columns show LDH activity expressed as percentage of total LDH content of the culture.

(**: p<0.01 compared to control cultures not treated with ethanol)

B. Inhibition of alcohol-withdrawal induced cytotoxicity by re-addition of ethanol and by treatment with different drugs

Columns represent LDH-release after 24 hours of the indicated treatment in primary cultures of rat cortical neurones pre-treated with 100 mM ethanol daily for 3 successive days. Control column shows LDH-release in cultures not adapted to ethanol. (*: p<0.05, **: p<0.01 compared to the alcohol-adapted and withdrawn group)

comprised to a large extent of NMDA-mediated components, while in slices from ethanol-naive animals, synaptic potentials were rather AMPA-mediated. In addition, when ethanol was superfused for one hour, a transient depression of the NMDA-induced depolarisation has developed, which vanished however, at the end of the superfusion period. Furthermore, the washout of ethanol caused a withdrawal-type increase in the responses to NMDA. Similarly, while the amount of non-viable cells detected via propidium iodide

(PI) labelling in hippocampal brain slice explants was significantly reduced in presence of ethanol, NMDAR-mediated increases in PI labelling was significantly higher in ethanol exposed groups after 24h ethanol withdrawal. These data also suggest that acute administration of ethanol has a minor neuroprotective effect but neurones became more sensitive to NMDA following long term ethanol exposure and withdrawal [70, 71].

Correspondingly, when cultures of rat cortical cells were chronically treated with ethanol (100 mM) the morphology of neurones were not altered, whereas obvious signs of neuronal damages and increased release of lactate dehydrogenase (LDH) were observed after 24 hours of withdrawal [11]. 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. (2A)) [72]. This kind of daily ethanol treatment produces a neuronal model of alcohol-adaptation similar to that of Hu and Ticku [73]. They used chronic but intermittent ethanol (50 mM) treatment (CIE, 12 h ethanol: 12 h withdrawal) providing a feasible *in vitro* model for studying the mechanism underlying the CIE-induced kindling-like phenomenon observed in humans [74, 75]. Furthermore, it may be useful for studying ethanol dependence and withdrawal symptoms in alcoholics who consume alcohol intermittently for an extended period. Further observations using this model are that the neuronal cell loss was reduced by re-addition of ethanol or by administration of NMDAR antagonists, whereas the GABA$_A$ receptor agonist muscimol was ineffective (Fig. (2B)). These conclusions support the conception that NMDARs play crucial role in the development of *in vitro* ethanol dependence and in alcohol-withdrawal evoked neurotoxicity. This hypothesis was further strengthened by the observation that NMDA responses and sensitivity of NMDARs for ethanol were enhanced after chronic ethanol pre-treatment [72].

In addition to the increased function of NMDARs, enhanced release of glutamate was also observed *in vitro* as well *as in vivo after* chronic ethanol exposure. Besides several factors (adaptations in GABA receptors, calcium channels, etc) the NMDARs are major contributors to increased glutamate release during alcohol withdrawl *in vivo*. 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 [76, 77]. This enhanced glutamate release may contribute to the further shift towards the excitatory dominance in the CNS after ethanol withdrawal. Furthermore, the up-regulation of the NMDARs can enhance the activity of the noradrenergic system [78] that may account for the instability of vegetative system seen in alcohol withdrawal and delirium tremens [1, 2].

It has been hypothesised that the same neuronal system including the mesolimbic dopaminergic pathway mediates the reinforcement for alcohol and for other addictive drugs like opiates or cocaine [79,80]. Indeed, ethanol has been reported to stimulate dopamine (DA) release in the nucleus accumbens (NA) [81] and electrophysiological studies have demonstrated a concomitant ethanol-induced increase in the activity of ventral tegmental dopaminergic neurones [82, 83]. On the other hand, systemic administration of NMDAR antagonists increases burst firing of dopaminergic neurones [84] and can stimulate release of DA in dopaminergic terminal areas suggesting that glutamate - acting through NMDARs - exerts a tonic inhibitory action on DA release in the NA [12, 85]. According to the model of Fadda and Rossetti [77], blockade of the NMDARs by acute ethanol

treatment disinhibits dopaminergic neurones via GABAergic interneurones 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 [86] due to decreased firing rate of dopaminergic neurones [87]. Thus, reduced dopaminergic functions seen after ethanol withdrawal may arise as a consequence of enhanced NMDA responses induced by chronic ethanol exposure.

All the above discussed findings suggest the possibility that increased NMDA mediated neurotransmission may consist the basis of both the overt signs (e.g. seizures, tremor etc.) and the affective or emotional disturbances (e.g. craving, dysphoria) seen after alcohol withdrawal. According to this view of the pathomechanism of alcohol withdrawal syndrome (AWS), NMDAR antagonists may be useful in the treatment for both physical and psychical signs of alcohol withdrawal.

EFFECT OF ETHANOL ON NMDA RECEPTOR SUBUNIT COMPOSITION

According to earlier reports it was hypothesised that chronic ethanol treatment leads to an increase in the number of NMDARs leading to facilitated receptor function [49], but chronic-ethanol-induced up-regulation of NMDARs has not been found in all studies [88,89]. According to the newly emerging view, the increased NMDAR function is presumably due to a differential up-regulation of the different NMDAR subunits. This conception is supported by several papers presenting evidence for altered NMDAR subunit composition due to chronic ethanol treatment. Notwithstanding, there is a disagreement in respect of the expression of each subunit. On the one hand, some authors reported no changes in subunit expression due to long-term ethanol exposure at all [90] and others found changes solely in the expression of the NR2A subunit [91]. On the other hand, there are several papers concluding that besides several other types of subunits and NR1 splice variant forms the expression of the NR2B is increased. Lots of *in vitro* studies showed increased NR2B mRNA levels, with no change in NR1 and/or NR2A subunit transcription in cultured cortical neurons following chronic ethanol administration [92-94] On the contrary, the levels of NR2B as well as NR2A and NR1 subunit proteins were reported to be increased in the cortex and hippocampus of rats or mice [95-101]. Furthermore, in cultured cerebellar granule cells, a delay in the 'developmental switch' of the NR2B for NR2A subunits was found resulting in higher NR2B and lower NR2A subunit levels [102]. Novel data concerning the expression of the different NR1 splice variants showed a marked decrease in the ratio of the N1 cassette holding and lacking splice variant mRNAs. However, the ratio of the 3 splice variants (N1, C1 and C2) was shown unaltered in cerebral cortex of rats chronically treated with ethanol [103].

In primary cultures of rat cortical neurones treated with ethanol for three consecutive days, we found that the maximal inhibitory effect of ethanol - similarly to the examined NR2B SSNAs - was significantly increased (Fig. (3A)). On the contrary, the efficiency of the non-subunit selective NMDAR antagonists was not changed. According
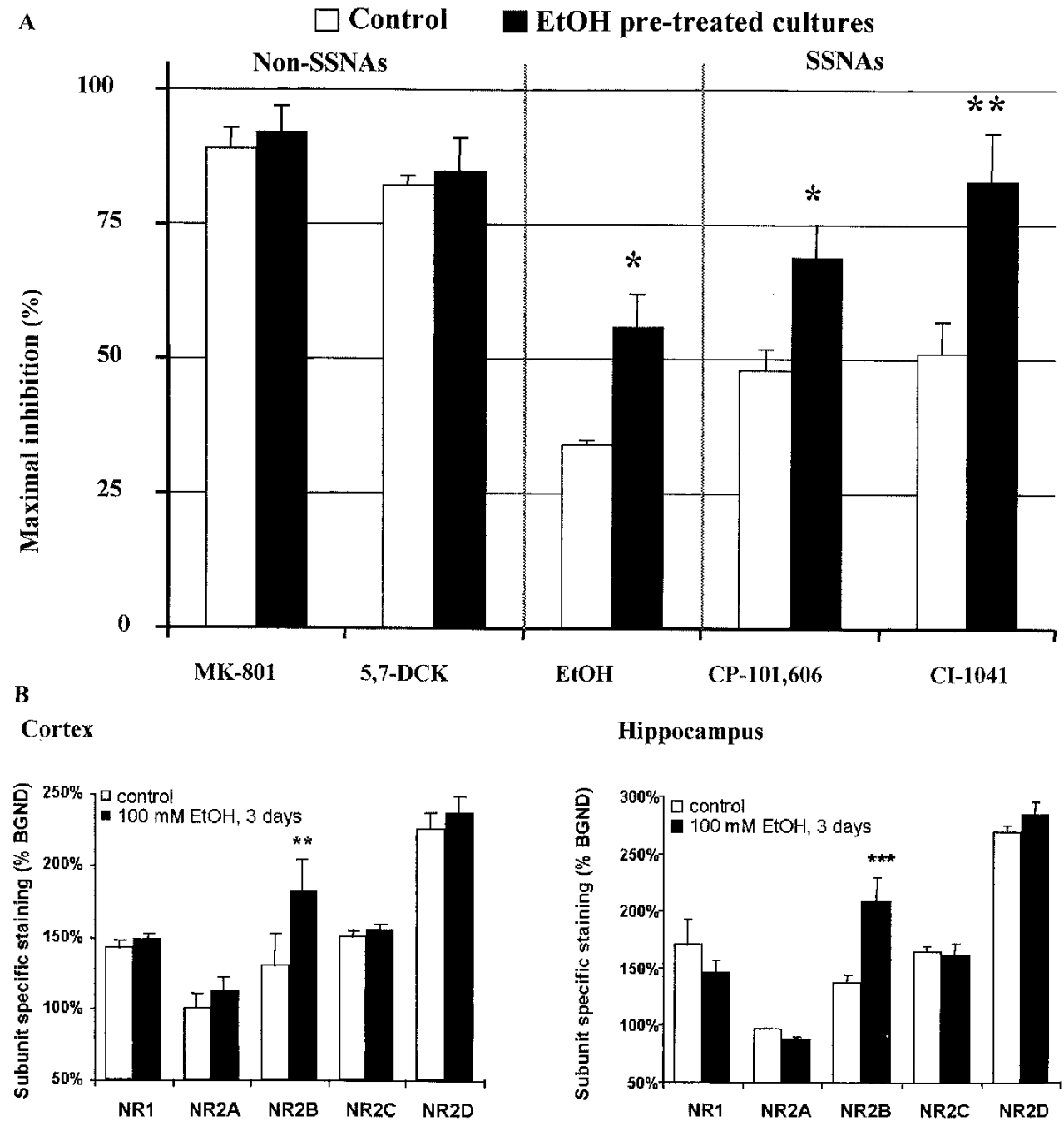
Fig. (3). Effect of ethanol on NMDA receptor subunit composition

A. Effect of chronic ethanol pre-treatment on the inhibitory potential of different NMDA receptor antagonists.

Maximal inhibitory effect of different NMDA antagonists on 40 μM NMDA evoked cytosolic Ca^{2+}-elevations in control (white columns) and 100 mM ethanol pre-treated (black columns) primary cultures of rat cortical neurones. (*, $P < 0.05$; **, $P < 0.01$ compared to control)

B. Effect of chronic ethanol pre-treatment on the expression of NMDA receptor subunits.

Expression of NMDA receptor subunit proteins was estimated by a flow-cytometry based immuno-cytochemical method in primary cultures of rat cortical and hippocampal neurones. Columns show the background related fluorescence values measured in control (white columns) and ethanol (100 mM) pre-treated (black columns) cultures. (**, $P < 0.01$; ***, $P < 0.001$ compared to control).

to these observations, a shift in subunit expression in favour for the NR2B subunit was supposed. Indeed, increased immuno-staining of the NR2B subunit proteins was observed in ethanol pre-treated cultures of rat cortical and hippocampal neurones (Fig. (3B)). Besides the NR2B subunit, the expression of the C1 and C2' cassette containing splice variant forms of the NR1 subunit proteins were also increased in these cultures, whereas the immuno-labelling of the NR2A, NR2C and NR2D subunits was not changed [104].

Although the mechanisms involved in alcohol induced changes in expression of the different NMDAR subunits are not yet identified and need further investigations, based on our observations, and considering that i) the deactivation time of NMDARs composed of NR2B subunits is longer than those bearing NR2A subunits [22]; ii) the deactivation rate is four times faster for receptors composed of NR1 subunit containing the N1 cassette than those lacking it [105, 106]; and iii) NMDARs assembled of NR1 splice variants containing C1 and/or C2 cassettes may form functionally more active ion channels [107], one can conclude that these changes in subunit expression due to long-term ethanol exposure may underlie the increased NMDAR functions and subsequently the enhanced sensitivity of ethanol treated cortical neurones to excitotoxic insults seen after ethanol withdrawal. Knowing that

glutamate is only one component of the neurotransmission processes in the CNS and data from *in vitro* experiments must be handled with exceptional caution when conclusions regarding manifestation of changes in a whole organism are drawn, alterations in NMDAR functions seem to play a critical role in the development of alcohol dependence, and might underlie the neuronal cell loss in certain areas of the brain during alcohol withdrawal.

NR2B SSNAS INHIBIT ALCOHOL WITHDRAWAL SYNDROME

In the early 90s, it was already hypothesised that NMDAR antagonists can block alcohol withdrawal induced seizures in ethanol dependent animals. Since then, extensive animal literature and preliminary clinical observations suggest that NMDAR antagonists are excellent candidates for the treatment of AWS, inasmuch as these compounds may attenuate not only the physical but also the affective and motivational components of AWS (see review [16]).

There are several different ways to inhibit NMDAR activation. So far, mostly the classic competitive and channel blocking NMDAR antagonists were tested and proved to be useful in *in vitro* or animal models of alcoholism. However, most of these compounds are psychotomimetic or sedative and produce severe CNS side

Table 1.   Some NR2B Subunit Selective NMDA Antagonists with Different Structures
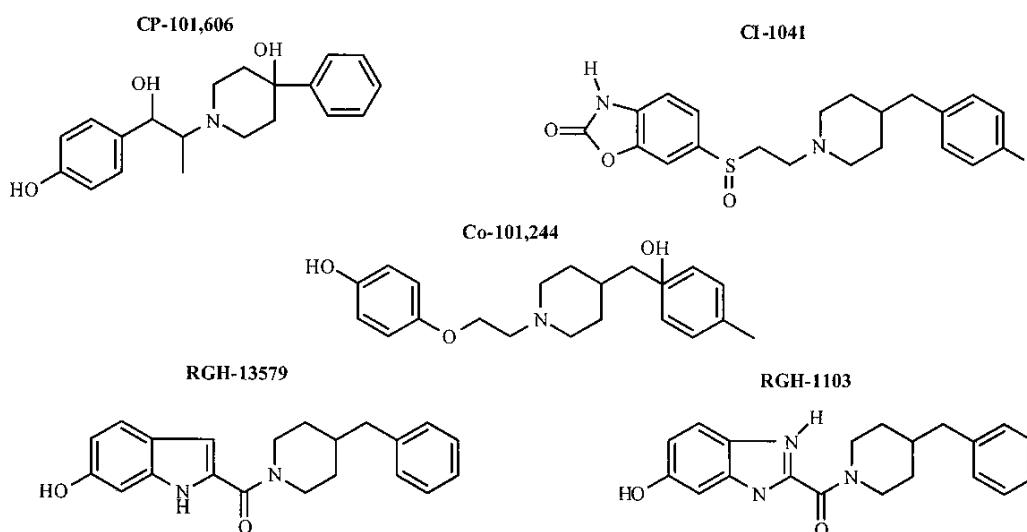
Compound	Inhibition of NMDA evoked Ca^{2+} responses (IC_{50} values)		Selectivity (NR2B vs. 2A)
	NR1/NR2A	**NR1/NR2B**	
CP-101,606	> 30 μM	30.0 ± 3.6 nM	> 1000
CI-1041	> 50 μM	8.4 ± 1.4 nM	> 5950
Co-101,244	> 30 μM	4.8 ± 0.7 nM	> 6250
RGH-13579	> 30 μM	19.9 ± 4.5 nM	> 1600
RGH-1103	> 30 μM	2.1 ± 0.4 nM	> 13600

Compounds were tested for their inhibitory activity on 100 μM NMDA induced calcium elevation in HEK293 cells stably expressing recombinant NMDA receptors [143]. IC_{50} values were determined and degrees of selectivity were evaluated as the ratio of IC_{50} values obtained in cells expressing NR1/NR2A and NR1/NR2B receptors.

Table 2. Inhibitory Effect on NMDA Evoked 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
e-ifenprodil	72 ± 2	0.475 ± 0.045	90 ± 14	3.8 ± 1.2
CP-101,606	58 ± 2	0.041 ± 0.005	89 ± 9	0.201 ± 0.061
CI-1041	70 ± 3	0.007 ± 0.001	82 ± 6	0.037 ± 0.008
Co-101,244	67 ± 2	0.024 ± 0.003	101 ± 5	0.206 ± 0.045
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 40 μM NMDA evoked cytosolic calcium elevation and on ethanol-withdrawal produced neurotoxicity in primary cultures of rat cortical neurones. IC_{50} and I_{max} values of the compounds were determined by fitting sigmoidal curves on data points using Microcal™ Origin® 6.0 data analysis software.

effects such as muscle relaxation, neurodegenerative changes, motor and learning impairment [108, 109] which make them unsuitable for pharmacotherapy. More encouraging approaches were performed with low affinity channel blockers like memantine or with NMDA antagonists acting on the glycine binding site (L-701,324) [110, 119] having more tolerable side effect profiles.

In recent years, a novel type of NMDAR antagonists, the NR2B subunit selective ones have received considerable attention. This type of compounds showed potency in models of neurodegeneration [112], Parkinson disease [113-116] and hyperalgesia [117-120]. It was also realised that this type of compounds lacks the serious side effects of the classic NMDAR antagonists' [121]. 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 SSNAs such as CP-101,606 (traxoprodil) [122, 123], Ro25-6981 [124], Co-101,244 [125] and CI-1041 [126] 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 [117 120, 127, 128].

The initial chemical leads of NR2B SSNAs - ifenprodil and eliprodil - were found effective in animal models of alcohol dependence. According to Malinowska *et al.* [129], ifenprodil potently reduced 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 [110]. This effect of eliprodil was achieved at doses, which by themselves did not alter the basal locomotor activity of untreated control animals. Similar results were observed in alcohol dependent male C57BL/6J mice [97]. The expression of spontaneous ethanol withdrawal signs (piloerection, jerk, tremor) occurring after 6 – 12 hours of the discontinuation of ethanol

treatment was suppressed by ifenprodil and was accompanied by marked up-regulation of the NR2B subunits in the limbic forebrain of ethanol treated mice. According to Kotlinska and Liljequist [130], eliprodil effectively reversed the reduction in extracellular DA level during ethanol withdrawal but only partially and not dose-dependently substituted for ethanol indicating that it has no discriminative stimulus properties similar to those produced by ethanol.

A causal relationship between NMDA antagonism and the anticraving properties of the recently launched agent acamprosate, which was originally developed as a GABAergic drug, has also been suggested [131-135]. It was observed that it reduced alcohol-consumption as well as the increase in extracellular glutamate level and hypermotility during ethanol withdrawal [136-138]. At least one of the biological mechanisms by which acamprosate achieves its therapeutic effects is thought to be its effect at the polyamine (spermidine) binding site of the NMDARs [139, 140].

Based on these observation, we investigated the effect of some NR2B SSNAs in ethanol treated cultures of rat cortical neurones. All the tested NR2B SSNAs (novel indole-2-carboxamide derivatives: RGH-13579 and RGH-1103 [141], CP-101,606, Co-101,244 and CI-1041) (Table 1.) potently and dose-dependently reduced the 24h withdrawal-evoked LDH release. One of the novel compounds, RGH-1103 was as effective as the most potent but non-subunit selective classic NMDAR antagonist MK-801. The inhibitory potencies of NR2B SSNAs for withdrawal-induced toxicity was in good linear relation with their effectiveness for inhibition of NMDA induced cytosolic calcium elevation (Table 2.)_[142].

SUMMARY

According to the recently emerged view of the pathomechanism of alcohol withdrawal syndrome, increased NMDAR function plays a central role in the development of alcohol dependence and manifestation of the withdrawal

symptoms. Furthermore, this alteration is, at least partly, due to an increase in the expression of the NR2B subunit. Experimental data indicate the therapeutic power of NMDAR antagonists since they effectively i) inhibit alcohol withdrawal associated hyperactivity and seizures, ii) prevent *in vivo* and *in vitro* neurotoxicity after alcohol withdrawal, iii) revert the reduced DA brain levels during withdrawal [80], and iv) reduce ethanol self-administration in genetically alcohol-preferring rats. Ifenprodil and eliprodil, the prototypes of the NR2B SSNAs potently inhibited ethanol withdrawal signs in animal models of alcohol dependence without discriminative stimulus properties. Novel NR2B SSNAs, similarly to classic NMDAR antagonists, potently inhibited the withdrawal-induced neurotoxicity *in vitro*, in ethanol pre-treated cultures of rat cortical cells. Based on these observations, the NR2B type of NMDAR subunits may be a potential drug target in treatment for alcohol dependence and withdrawal. Considering that NR2B SSNAs have advantages over the classic NMDA antagonists in terms of their side effect profile, these compounds can be promising therapeutic candidates for the pharmacotherapy of alcohol withdrawal syndrome. To prove the *in vivo* efficiency of these compounds in treatment for physical and/or affective components of AWS, further investigations are needed.

ACKNOWLEDGEMENTS

ABBREVIATIONS

AWS	=	Alcohol withdrawal syndrome
CIE	=	Chronic but intermittent ethanol treatment
DA	=	Dopamine
NA	=	Nucleus accumbens
LDH	=	Lactate dehydrogenase
NMDA	=	N-Methyl-D-aspartate
NMDAR	=	N-Methyl-D-aspartate receptor
PI	=	Propidium iodide
SSNA	=	Subunit selective NMDAR antagonist

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