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ORIGINAL PAPER

GluN2B Subunit of the NMDA Receptor: The Keystone of the Effects of Alcohol During Neurodevelopment

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Abstract
The glutamatergic system plays a central role in both the acute and chronic effects of ethanol. Among all the glutamate receptors the ionotropic NMDA receptors are crucial because of their role in synaptic plasticity. A large body of evidences suggests that short-term and long-term effects of ethanol may change synaptic plasticity via an alteration of the expression of the GluN2B subunit, one constitutive element of the NMDA receptor. The present review is focusing on the role of the GluN2B subunit after ethanol exposure during early life (in utero and adolescence) and also at adulthood. The roles of other NMDA subunits are also discussed in the context of the increasing evidence that the ratio of the different subunits, such as GluN2A-to-GluN2B, seems to better reflect the effects of ethanol and to explain how ethanol exposure can have short lasting and long lasting effects on synaptic plasticity, cognitive processes and some of the ethanol-related behaviors.

Keywords GLUN2 subunit · NMDA · Alcohol · Glutamate · In utero · Adolescence

Introduction

The identification of the ethanol targets in neurons is a long story. After the membrane theory aimed at explaining anesthesia [1], neuronal membrane receptors were identified in the 80s as ethanol targets such as $GABA_A$ and NMDA receptors (NMDA-Rs) [2]. In the 90s a NMDA-R like complex has been identified and has also been shown to be the target of both acute and chronic ethanol exposure [3]. Numerous behavioral effects of ethanol are explained by its inhibitory action on NMDA-Rs that are involved in both the acute and chronic effects of ethanol. A main challenge during the past three decades has been to understand how the effects of ethanol are mediated through an action on specific subunits of the NMDA-Rs and especially in the context of early life ethanol exposure (in utero life and adolescence) and in the development of alcohol use disorder (AUD, abuse and addiction). An important role of NMDA-Rs in the effects of ethanol is through its action in ethanol-induced excitotoxicity and in ethanol-induced disturbances of synaptic plasticity. In the present review, a specific focus will be done on the role of the GluN2B subunit of the NMDA-Rs that has been shown to be involved in the long-term effect of early life ethanol exposure during the in utero life and adolescence.

Glutamate is an amino-acid that mediates the excitatory transmission in a majority of synapses throughout the central nervous system (CNS; [4]). Glutamate exerts its excitatory effects via a large family of membrane receptors including both ionotropic and metabotropic types of receptors. The ionotropic glutamate receptors (iGluRs) are tetrameric cation channels subdivided into four families: AMPA receptors (AMPA-Rs), kainate receptors, NMDA receptors and the orphan delta receptors (GluD1 and GluD2). A first classification was based on the sensitivity of the receptors to different analogues of glutamate: *N*-methyl-D-aspartate for NMDA-Rs; kainic acid for kainate-Rs and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid for AMPA-Rs. Functionally, a great amount of work has been performed since it was found that iGluRs (NMDA-Rs and AMPA-Rs) are mediating brain plasticity through modulation of synaptic plasticity, the long-lasting changes in synaptic transmission suggested to be part of cellular mechanisms underlying higher cognitive functions such as learning and memory.

NMDA-Rs are a family of receptors per se because of an interesting diversity in subunit composition leading to

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different electrophysiological, biophysical, and pharmacological properties [5]. In addition, the properties of the NMDA-Rs are different according to the brain area; these properties evolve during maturation and can be further modulated through experience and or specific pharmacological environment such as exposure to ethanol. The influence of the environment on NMDA-Rs properties may thus lead to pathological situation such as addiction. Therefore and opposite to the physiology, several neuropsychiatric or neurological diseases including addictions, are linked to NMDA-Rs dysfunctions [6]. In this context, drug addiction including ethanol addiction is suggested to occur at least partly through modulation of NMDA-Rs properties and then through the alteration in synaptic plasticity, in which NMDA-Rs have a key role, notably in the brain structures of the mesolimbic circuit. Indeed, drug addiction is often referred to as a pathological memory [7]. There is today an increasing amount of literature suggesting that NMDA-Rs play a major role in mediating the effects of ethanol on behavior and on the development of AUDs [8, 9]. Interestingly, such literature points out an important implication of the GluN2B subunit of the NMDA-Rs as one central regulator of addiction to different drugs, leading then to the proposal that selectively targeting this subunit may represent an interesting alternative therapeutic approach, although other targets may exist (GluA3, NMDA-Rs glycineB site or mGlu5, mGlu2/3; see [8, 10]). Thus, increasing our understanding in the physiology and physiopathology of the NMDA-Rs at synaptic levels in the mesolimbic circuit should help elaborating new strategies to maintain or rescue homeostasis in this circuit and to treat or prevent addiction.

The NMDA Receptors Family: A Matter of Subunit Composition

All subunits of iGluRs have similar molecular structure made of four domains: (i) a C-terminal intracellular domain (CTD) involved in synaptic targeting, (ii) an N-terminal extracellular domain (NTD), (iii) a membrane-proximal ligand-binding domain (LBD) connected to (iv) a transmembrane domain (TMD) which form the ion channel. NTD and LBD domains form the extracellular portion of the receptor and present a bilobate structure that binds ligands in the interlobe space. iGluRs assemble into homo- or heterotetramers and NMDA-Rs are either di- or tri-heteromeric complexes. Cloning studies revealed so far three subfamilies of NMDA-Rs subunit (GluN1, GluN2 and GluN3) with seven different subunits: GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A and GluN3B. NMDA-Rs are composed of subunits of the NR1 family which encompassed eight isoforms (GluN1-1a to GluN1-4a and GluN1-1b to GluN1-4b) produced by alternative splicing of a single gene named *Grin1* in the rat, and with subunits of the GluN2 family which comprise four members produced by four distinct genes (*Grin2a-d* in the rat). GluN3 subunits are encoded by two separate genes (*Grin3a-b*). These latter subunits are less characterized but the two co-agonists glycine and D-serine also binds to these GluN3 subunits [11]. In addition to the well-known NMDA-Rs another complex of proteins called NMDA receptor-like complex has been shown to contain ligand-binding sites characteristic of NMDA-Rs expressed on CNS neurons. The proteins of the NMDA-R like complex are the 71 kDa glutamate-binding protein (GBP), the glycine-binding (GlyBP), and the carboxypiperazinyl-propylphosphonic acid-binding protein [3, 12].

The GluN1 subunit isoforms are differentially expressed within the CNS and the functional significance of such distribution remains unclear. Importantly, the GluN1 subunit carries the binding site for glycine or D-serine, two different positive co-agonists of NMDA-Rs [13]. The native NMDA-Rs either di- or tri-heteromeric are never composed of subunits from one family only and although the composition and stoichiometry of such receptors has still to be clearly identified, there is ample evidences that some NMDA-Rs are formed of two GluN1 subunits associated with two different GluN2 subunits which contain the binding site for glutamate. Some NMDA-Rs however are thought to be composed of GluN1, GluN2 and GluN3A subunits [5]. The difference in subunit composition arises from the different size of the C-terminal of each subunit which importantly determines an intracellular domain involved in both NMDA-Rs trafficking and its interactions with different intracellular biochemical pathways. The difference in subunit composition, and principally in GluN2 subunits, has major consequences in the receptor's properties. Notably a first consequence is the profile of expression during brain maturation with GluN2A expression rising almost constantly from birth to adult age ending in a generalized expression throughout the brain while GluN2B expression is high at birth and decreases progressively after the first postnatal week in rats to reach a low level at adult age with a pattern of expression mainly located in the forebrain. GluN2D is high at birth then decreases rapidly and strongly to be almost expressed only in some part of the midbrain at adult age whereas GluN2C appears gradually from postnatal day 10 with a final expression restricted to the cerebellum and the olfactory bulb. Concerning the GluN3 subunits, the A isoform peaks early in postnatal life and decreases progressively until adult age while the B isoform slowly increases during maturation to be finally highly and preferentially expressed in the motor system. The different properties of the NMDA-Rs are thus expressed throughout the development of the brain. Finally at adult age, the predominant isoforms are GluN2A and GluN2B suggesting they play a major role in CNS function. Importantly, GluN2A and GluN2B subunits are particularly expressed in the hippocampus and the cortex, two regions known to

express high level of synaptic plasticity and to play important role in learning and memory. Furthermore, the different subunit composition of the NMDA-Rs endows it with specific and distinct single channel and kinetic properties, pharmacological characteristics to agonists, antagonists and co-factors [5].

Site of Action of Ethanol

The question of the specific site of action of ethanol is quite old and today it is largely agreed that there is no specific receptors responsible for the large ensemble of effects of ethanol in contrast to other drugs of abuse which all show specific membrane receptors dedicated to the drug's effects. Probably the first suggested site of ethanol actions was the bilayer lipid cellular membrane with a potential role as disruptive agent due to ethanol biophysical properties. In the late 70s to early 80s, a large literature was issued regarding ethanol effects on lipid membranes using notably synaptosomes as experimental model. In this era the idea was that ethanol primary effect relied on an alteration of membrane structure by "fluidizing" cell membranes through its intercalation between some fatty acid chains of the membranes increasing then the lateral mobility of some components of the membrane [14]. In consequence, the physiological effects of ethanol were due to this fluidization and tolerance to ethanol was supposedly based on resistance to this effect. In this context, it was reported that chronic ethanol exposure in adult male rats modified the composition of synaptosomal membranes with an increase in oleic acid and in phosphatidylcholine while that of arachidonic acid and in phosphatidylethanolamine were decreased. Furthermore, a correlation was found between the changes of fatty acids in the brain and that in the serum of ethanol-exposed rats [15]. However, chronic ethanol treatment in mice had no effect neither on total phospholipid levels nor phospholipid acyl composition in different subcellular fractions although significant increases in cholesterol content and cholesterol-to-phospholipid ratios were observed in plasma membranes of synaptosomes [16]. Thus, changes in lipid composition of the membrane were suggested to only partly account for an adaptation to ethanol-induced membrane disordering [17]. In this context, Michaelis and his colleagues [18, 19] fully participated to the expansion of this knowledge showing that ethanol affected membrane lipid organization or protein complexes such as the glutamate binding protein in a concentration dependent manner. Thus, up to 2 mM ethanol increases rigidity of the membrane of egg lecithin and bovine brain phospholipid liposomes while higher concentration increases fluidity of the bilayer [18]. Ethanol at low concentration decreased fatty acid chain motion whereas at higher concentrations (> 400 mM) it increased lipid motion [19]. Recently, the "fluidizing" role of ethanol has been measured using synaptosomal membranes obtained from chronic ethanol treated rats for 60 days for example [20] showing a decrease in cholesterol-to-phospholipids ratio, still suggesting disturbances of motion of membrane components are part of ethanol effects.

At functional level, ethanol is known to have both stimulatory and depressant effects on CNS activity. Low doses of ethanol have stimulatory effects expressed as increased behavioral arousal associated with mild or no intoxication and supposed to result from a dis-inhibitory action. In contrast, depressant action of ethanol on CNS and on behavior is expressed with moderate to high doses of ethanol and may result from a decrease in excitability of neurons. Such decrease can be obtained with either an activation of inhibitory transmission and/or an inhibition of excitatory receptors, or both. Among the iGluRs, NMDA-Rs show several specific features: they require either glycine or D-serine as co-agonists according to their subunit composition [21]; they demonstrate a high Ca^{2+} permeability; they are blocked in a voltage-dependent manner by physiological levels of Mg^{2+}, and show slow activation and inactivation kinetics compared to the faster AMPA/kainate-Rs. At resting membrane potential, ambient Mg^{2+} blocks NMDA-Rs and this is released once the cell is depolarized typically upon AMPA/kainate-Rs activation. Consequently, an influx of Ca^{2+} occurs in the depolarized cell which will trigger a variety of intracellular pathways either involved in synaptic plasticity or in excitotoxicity according to the final intracellular concentration of free Ca^{2+}. Therefore, depressant effects of ethanol are partly due to its inhibitory effects of NMDA-Rs. In parallel to the question of ethanol's effects into the membrane, research on the effects of ethanol on CNS was developing and it was reported in 1978 the description of a supersensitive state to glutamate during ethanol addiction which supposedly was contributing to the signs of ethanol withdrawal as mentioned by the authors [22]. Later, the same research group linked the effects of ethanol on the membrane with disturbances of some membrane proteins associated with nerve cell membranes such as receptors, taking advantage of the recently described putative excitatory neurotransmitter L-glutamic acid. Indeed, they confirmed their previous experiments combining in vitro and in vivo approaches with synaptosomes and chronic ethanol treatment in rat, respectively. With such approaches they reported evidences that sensitivity to glutamate was increased in both models [18, 23]. In addition, low concentration of ethanol (9.4 mM) diminishes glutamate-induced depolarization of synaptic membranes while 93.7 mM increases passive influx of the marker used to measure such effect of ethanol [19]. Once the glutamate receptor was defined as one target for ethanol it was necessary to determine the exact site of action of ethanol on NMDA-Rs.

NMDA-R Subunits and Ethanol-Binding Site

Several studies were conducted to find if a binding site exists for ethanol and to determine the exact position of such an ethanol "pocket" into the NMDA-R structure. The precise mechanism by which ethanol inhibits the NMDA-R is still unknown but ethanol probably does not interact with any of the already known modulatory sites of the receptor nor it acts as an open channel blocker [24]. GluN1 subunit is mandatory since it contains domains that confer glycine binding and ion selectivity. Therefore several studies tested the effects of amino-acids substitutions with site-directed mutagenesis in transmembrane domains 3 (TM3) or within TM3 and TM4 in recombinant receptors. Substitution of alanine for a phenylalanine residue in TM3 of the GluN1 reduced ethanol sensitivity [25]. Further studies [26], indicated that additional discrete sites within TM3 and/or TM4 regulate ethanol sensitivity of NMDA-Rs without, however, being a proper site of fixation [27].

Concerning other subunits, using constitutively open receptors after mutation of an alanine in TM3 of NMDA subunits, ethanol was shown to induce its effects on NMDA-Rs function at a distal site from agonist binding ones and preferentially via interaction with GluN2 subunits [28]. GluN2A seems particularly involved in sensitivity to ethanol [29]. A single amino-acids substitution in TM4 of the GluN2A subunit reduced inhibitory effects of ethanol [30]. Furthermore, two adjacent phenylalanines in TM3 of GluN2A possibly regulate sensitivity and ion channel gating effects of ethanol [31]. It is further suggested that up to four sites for ethanol co-exist on the NMDA-R each consisting of 5 amino-acids and located at M3–M4 domain intersubunit (GluN1–GluN2A) interfaces [32]. Interactions between different sites into TM3 and TM4 have been also reported within GluN2B subunit although without forming a unitary site of ethanol action [33]. However, NMDA-R affinity, channel gating and ethanol sensitivity seem to depend upon the amino-acid F637 of GluN2B but without occupying a critical volume at this position unlike the cognate site in GluN1 subunit [34]. The NTD part of NMDA-R does not seem to be a primary target of ethanol [35] although it is affecting sensitivity. Finally ethanol inhibits NMDA-Rs by reducing channel opening probability and mean open time via interaction with specific residues within TM3 and TM4 of GluN2A and GluN2B subunits [36]. Different sites although all in TM3 and TM4 in GluN2A and GluN2B subunits have been reported accompanied with a modulation of ethanol sensitivity and ion channel gating [37]. Indeed, despite the highly-conserved M domain sequences in GluN2A and Glun2B subunits showing similar ethanol sensitivity, it is suggested that the way these two subunits interact with GluN1 to regulate ethanol sensitivity and NMDA-R kinetic may differ [38].

NMDA-R Subunit and Ethanol Exposure

Although alcohol has no dedicated receptor within the CNS, it modifies several neurotransmitter systems from amino acids ones (excitatory and inhibitory) to neuromodulators from different chemical family, such as catecholamines, indolamines…. We previously mentioned the early reports of Michaelis and his colleagues [18, 19, 23] regarding the possible interaction between glutamate and ethanol at cellular membrane level. In these studies, ethanol's effects on the affinity of glutamate binding on synaptic plasma membranes were explored. At that time, is was also investigated the glutamatergic synaptic transmission as well as the depressive effects of alcohol and chemically related compounds at the lobster neuromuscular junction or in spinal cord neurons [39, 40] for examples. The levels of several types of neurotransmitters in the brain after maternal ethanol consumption [41] or in alcohol-preferring rats [42] were also part of the research on ethanol's effects. It is not before the end of the 1980s however that studies were published regarding the interaction between NMDA-Rs and ethanol in different brain structures, at a time when pharmacological specific agonist (NMDA) and antagonist (MK-801) were on the market for preclinical research. Prenatal ethanol exposure, *ex vivo* acute ethanol exposure, in vivo chronic exposure, seizure activity were among the models tested and the hippocampus was appreciated by the scientists, probably because memory processes were greatly studied in this region. Thus, it was rapidly described that ethanol reduces glutamate binding, inhibits NMDA-induced currents and NMDA-induced noradrenaline and acetylcholine releases; and interact with kindling or seizure activity induced by excitatory agents [2, 22, 43].

Alcohol Exposure and NMDA-Rs at Adulthood

Regarding the effects of ethanol on glutamatergic neurotransmission and specifically on iGluRs, it is acknowledge that NMDA, AMPA and Kainate-Rs antagonists are all capable of attenuating alcohol-related drinking behavior although metabotropic glutamate receptors (mGlu) may also have a role to play [44]. Concerning NMDA-Rs, there are current accumulating evidences that both GluN2A and GluN2B subunits are important actors in the effects of ethanol and that the magnitude of ethanol's inhibition of NMDA-Rs is influenced by several factors including subunit composition [45] and the developmental status of the brain [46]. Indeed, it was shown that inhibitory effects of ethanol on NMDA-Rs can be mediated through interaction with GluN2B subunits in the dorsomedial striatum and in bed nucleus of the stria terminalis [47, 48]. However, the role of GluN2B in the inhibitory effects of ethanol on NMDA-Rs can not be generalized in the brain since it has been shown in the lateral orbital

frontal cortex that at high concentrations (66 mM), ethanol reduced NMDA but not AMPA-mediated excitatory postsynaptic currents and this effect was maintained in the presence of the selective GluN2B antagonist Ro-25-6981 [49]. Regarding the developmental status of the brain, NMDA-Rs in adolescent brains appear more sensitive to inhibition by ethanol [50] and in some brain areas this appears to be correlated with the level of expression of GluN2B subunits that are highest during early periods of development [46], but see [51]. Other studies on the ethanol sensitivity of recombinant NMDA-Rs have shown for example that GluN3a co-expression prevented the enhancement of ethanol's inhibitory effect on receptors composed of GluN2A but not GluN2B subunits [52]. At the behavioral level, the interaction between the Fyn kinase and the GluN2B subunit has been shown to mediate the acute sedative effects of ethanol [53].

During repetition of ethanol exposure, many studies converge on the idea that NMDA-Rs function increases mainly as a compensatory response due to an almost chronic blockade of NMDA-Rs throughout the exposure. Thus, glutamatergic neurotransmission is increased during withdrawal from ethanol in several brain structures [22].

The NMDA-R like complex is a target of both acute and chronic actions of ethanol on CNS neurons. Ethanol (5–100 mM) inhibits the glutamate and glycine-induced activation of the binding of [^{3}H]thienylcyclohexylpiperidine, an NMDA-R channel blocker, to the purified NMDA-R like complex [54]. Chronic exposure to ethanol in animals increases the expression of GBP, GlyBP and carboxypiperazinyl-propylphosphonic acid-binding proteins in synaptic membranes isolated from brains [3]. Interestingly, in cortical neuronal cultures from rat brain exposed to 100 mM ethanol for 72 h, the expression of both GBP and GlyBP is increased and this up-regulation is mediated through an increase in gene transcription [55]. Chronic ethanol exposure also induces a concomitant increase in GluN1 and GluN2B [54]. Interestingly, chronic ethanol exposure increases the half-life of GluN1 mRNA in fetal cortical neurons [55] and this half-life returns to control values when new protein synthesis is inhibited in ethanol-exposed neurons [56], highlighting the importance of proteins in ethanol-mediated stabilization of GluN1 mRNA. Thus cis–trans interactions are an important regulator of the ethanol-mediated stability of GluN1 mRNA.

In a model of neuronal adaptation to repeated exposures to ethanol, i.e., ethanol sensitization, it was reported that GluN2B subunit mRNA levels in the dorsal CA1 hippocampus area are increased exclusively in resistant mice [57] whereas another study revealed that GluN2A protein levels were decreased in the same population [58]. The effects of chronic ethanol exposure studied with culture of hippocampal and cortical neurons or with brain slices showed an upregulation of NMDA-Rs function associated with synaptic targeting for both GluN1 and GluN2B subunit but not for the GluN2A subunit. Interestingly, AMPA-Rs currents or responses to AMPA-Rs antagonist were unaltered suggesting a site-specific effect of ethanol on NMDA-Rs [59–61]. Furthermore, it has been shown that intermittent exposure to ethanol was more deleterious than chronic exposure probably due to the repetition of discrete withdrawal periods between each transient exposure. In such cases, GluN2B subunit expression was persistently altered [62]. GluN2B subunit increases in the nucleus accumbens (NAc) after repeated administration of ethanol [63]. More specifically, short lasting ethanol vapor inhalation increases NMDA-Rs current in NAc cells of the direct pathway with concomitant increases in the NMDA-dependent form of synaptic plasticity called long-term potentiation (LTP), but it decreases NMDA-Rs current in the NAc cells of the indirect pathway with Long-Term Depression, the functional opposite of LTP, being favored in this pathway [64]. In consequence, it has been suggested that short lasting exposure to ethanol vapor inhalation increases alcohol consumption via an increase in GluN2B subunit activity which in turn increases LTP in the direct pathway [65]. However, it is not known why the NMDA-dependent form of LTD is occurring simultaneously in the indirect pathway. Repeated daily administration of ethanol induces a long-lasting increase in the activity of GluN2B containing NMDA-Rs in the dorsomedial striatum but not in its dorsolateral part in rat, accompanied with an increase GluN2B phosphorylation and membrane expression of the subunit [47]. Importantly one consequence of such GluN2B-related hyperexcitability state would be to cause a significant increase in consumption since in contrast, if GluN2B subunit is blocked experimentally in the same structure, ethanol consumption in chronically exposed rats decreased as well as ethanol-induced reinstatement of ethanol seeking [47]. Still regarding GluN2B subunit involvement in the effects of ethanol exposure, a recent study tested the effects of only few repetition of ethanol administration on the NMDA-dependent forms of LTP and LTD as recorded in hippocampus slice of young adult rats. This study demonstrated that an increase in GluN2B subunit activity occurs as soon as only two injections of ethanol and that GluN2B overexpression leads to the blockade of NMDA-LTD. This interesting selective effect of ethanol on NMDA-LTD has been shown to last 6–8 days and to elicit cognitive impairment during this period of time [66]. Besides the NAc, the striatum and the hippocampus, ethanol also alters NMDA-Rs in different frontal cortical areas. In the mPFC, protein levels for GluN1, GluN2A and GluN2B subunits are increased after chronic ethanol exposure and this effect lasted up to 2 days of withdrawal [67]. A chronic intermittent exposure had similar transient effects on subunits expression with, however, a more persistent increase in NMDA-Rs function. Importantly, such effects of ethanol on NMDA-Rs activity

and subunits expression were accompanied with an impaired behavioral flexibility, an mPFC-dependent task. Similarly, NMDA-Rs activation and mRNA levels for GluN1 and GluN2A subunits but not for GluN2B were increase in the mOFC after chronic intermittent exposure and again, these changes were accompanied with an increase in compulsive-like behavior measured as the willingness to drink ethanol despite the delivery of an electric shock [68]. Importantly, such changes in NMDA-Rs and subunits were not found in mPFC or in dorsal striatum [69]. In contrast, chronic intermittent exposure to ethanol decreases GluN2B levels in lOFC, with an increase in neuronal excitability that lasted up to 10 days after withdrawal, an increase in AMPA-Rs current and an enhanced long duration form of LTP in this brain region [70]. In addition, ethanol inhibition of vBNST neurons that project to the VTA is expressed specifically via NMDA-Rs containing GluN2B subunit revealing how ethanol can modulate the VTA dopaminergic system [71]. Interestingly, other functions that cognitive ones are also concerned by ethanol exposure and by a role for GluN2B subunit. For example, ethanol inhibited spinal NMDA-induced pressor responses via an increase in phosphorylated form of GluN2B subunit leading to acute tolerance to inhibition by ethanol of NMDA receptor function [72]. At the genetic level also a particular role for GluN2B containing NMDA-Rs is acknowledge since in brains from alcoholics the *Grin2b* gene was upregulated (although with *Grin2d*) [73].

Altogether, the results mentioned above strongly suggest that repeated exposure to ethanol, either chronic or intermittent, enhances NMDA-Rs function through modulation of the different subunits of the receptor and probably and more specifically the GluN2B subunit. In addition, such changes in GluN2B subunit expression and NMDA-Rs activation is brain area-dependent and always accompanied with equivalent changes in synaptic excitability and/or synaptic plasticity, i.e., LTP and LTD. And importantly, most of the reported changes have been associated to some ethanol-related behaviors in animal models. Furthermore, humans with AUD also tend to show greater NMDA-Rs function [74].

Alcohol Exposure During Fetal Life and NMDA-Rs in Offspring

Another important period of ethanol exposure to be studied is during early brain development, i.e., in utero life. Because of the importance of this time period and of the known irreversible deleterious consequences of ethanol exposure during this period on cognitive function such as learning and memory, many studies had focused on describing the effect of ethanol on synaptic plasticity within the hippocampus. Preclinical studies that investigated changes in NMDA-Rs after early life ethanol exposure reported varying results probably due to the different animal species used (rat, mice or guinea-pig for most of them), the age at the evaluation periods (neonate, juvenile or adult offspring), the time when ethanol exposure was performed (during only one human equivalent trimester of gestation, throughout gestation, during pre and postnatal periods) and the way ethanol was administered to the dams (gavage, liquid diet…). Nonetheless, a large body of evidence exists regarding NMDA-Rs subunit expression and changes in pharmacological properties of the receptors. An early study describing the consequence of fetal alcohol exposure on NMDA-Rs subunits is maybe that of Iqbal et al. [75] who reported that GluN1 mRNA levels were increased in both CA3 and CA1 areas of the hippocampus of near term guinea-pig fetuses after a chronic prenatal ethanol exposure. In rat, a similar result was found in the hippocampus at postnatal days (PND) 7 and 14 [76] while GluN1 mRNA levels were decreased in adult hippocampus [77] after a pre- and postnatal exposure to ethanol. More recently, the same research group using the same model of ethanol exposure found a decrease in GluN2A and GluN2B mRNA levels with a non-significant decrease in GluN1 mRNA in hippocampus CA1 area of adult male offspring [59]. Interestingly, an innovative series of study revealed long-lasting abnormal modification of breathing activity in rat offspring that have been exposed to ethanol throughout gestation and lactation [78], leading also in brainstem respiratory network plasticity disturbances [79]. Here too and in accordance with forebrain areas, these effects of ethanol were accompanied with a reduced level of GluN2B mRNA in the medulla (Fig. 1).

Although measuring mRNA levels is interesting, a more informative measurement in terms of synapse functionality is probably the level of NMDA-Rs protein expression after ethanol exposure since influx of calcium through this receptor is highly dependent upon its subunit composition [5]. Specifically, the amount of calcium influx through the NMDA-R is larger in GluN2B containing receptor compared to GluN2A containing ones. Furthermore bidirectional synaptic plasticity is determined by the postsynaptic calcium concentration. Thus, it is of importance to know the effects of ethanol exposure on NMDA-R subunit expression. Concerning GluN1 subunit expression, some found it to be increased in the dentate gyrus (DG) of the hippocampus [80], whereas others did not find any changes [81]. The latter authors reported also no changes in GluN2A and GluN2B subunit expression in the DG. After prenatal ethanol exposure up to postnatal day 9, GluN2A was increase at PND 10 in the hippocampus without changes in the cortex [82]. If exposure was limited to the sole postnatal period in rat GluN2A was increased in the cortex at PND 21 [83]. Interestingly, these authors [82, 83] never reported any alteration in GluN1 and GluN2B expression levels in either brain regions, hippocampus or cortex, and after both pre- and

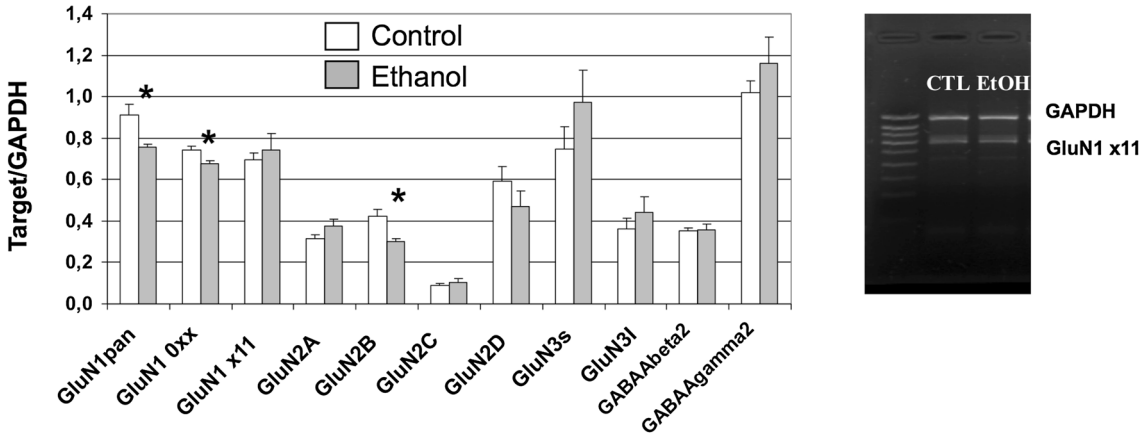

Fig. 1 mRNA levels for different targets of interest in the brainstem respiratory areas of juvenile rats. Animals were exposed to ethanol during gestation and lactation periods and the central respiratory network activity was recorded at postnatal days 8–10 in a transverse brainstem rhythmic slice (see [74]). mRNA levels for different NMDA (GluN1pan: all GluN1 splice variants); GluN1 0XX and GluN1 X11; GluN2A, GluN2B, GluN2C, GluN2D, GluN3s and GluN3l: GluN3 short and long variants; and GABA (β2 and γ2) receptors subunits were evaluated in these slices. On the right side is an example of result obtained for the atypical GluN1 X11 subunit (a specific splice variant of the GluN1 subunit), showing a slight increase (unpublished data from Pierrefiche and Naassila). See also [66] for results obtained with the same subunits but in the hippocampus

postnatal exposure or only during the human equivalent of the third trimester. Others, however, found that GluN2B was decreased at PND 7 in rat or in adult mice accompanied with a decrease in PSD95–GluN2B complex association [84] whereas GluN2B increased in some regions of the prefrontal cortex [85]. Interestingly, GluN2B expression was reduced in the DG [86] while it was increased in CA1 area [59], revealing the substructure alterations induced by ethanol. Finally, learning deficits in hippocampus-dependent tasks in adult rats after pre and postnatal ethanol exposure was accompanied by dysregulation in hippocampal gene expression through a significant induction of glutamate-related genes including those for GluN2A to D subunits [87].

An additional and more precise level of analysis for the expression of subunit is to take into account the location of the NMDA-Rs, i.e., synaptic versus extrasynaptic (perisynaptic or non-synaptic) location. This is particularly relevant since GluN2A and GluN2B subunits are believed to be differentially distributed between these two compartments and that the GluN2A-to-GluN2B ratio governs some aspect of plasticity properties of the synapses [5]. In this context, synaptic and non-synaptic fractions of the hippocampus DG showed a decrease in GluN2B subunits and an increase in C2-containing GluN1 and GluN3A subunits at the synapse [86] after prenatal ethanol exposure in mouse. Importantly, GluN1 and GluN3 subunits are poorly permeable to calcium while GluN2B is highly permeable to calcium. Therefore, such changes in subunit expression will eventually modify the plasticity of the ethanol-exposed synapse. Similarly, using subcellular fractionation it has been reported that after early life ethanol exposure GluN1 levels in synaptosomal membrane was increased without changes in GluN2A and GluN2B in hippocampus DG [84]. In addition, GluN1 and GluN2B levels were unchanged in whole tissue homogenates while GluN2A was reduced. Finally, PSD-95 associated pools of receptor subunits showed no changes for GluN1 or for GluN2A but a decrease for GluN2B [84]. Altogether, there is possibly an increase in GluN1 subunit expression accompanied with a decrease in GluN2B subunit at the synaptic level in the DG. Concerning the GluN1 subunit, it appears that if the increase in this subunit occurs, it is probably quite delayed since it does not happen at early ages that have been investigated (PND 7, 10 and 21) [82, 88]. Because of these rearrangements of the NMDA subunits at the synapse in the DG, we may anticipate that NMDA-dependent LTP and/or LTD should be altered mainly due to the decrease in GluN2B. Interestingly, in the CA1 area of the hippocampus, it has been reported an increase of GluN2B subunit in the synaptic compartment with a tendency for GluN2A to decrease after pre- and postnatal ethanol exposure [59]. Here, the authors suggest that such increase in highly permeable calcium subunit would lead to an increase in NMDA-dependent LTD and a concomitant decrease of LTP magnitude. Importantly, the different studies demonstrated the need for measurements to be performed at the substructure level to understand the differential effects observed in the different areas but also when possible to analyze synaptic versus extrasynaptic compartments to better understand the reorganization of the synaptic elements and the fate of NMDA-LTP and NMDA-LTD after ethanol exposure.

Is GluN2A/GluN2B Ratio an Important Factor in the Effects of Ethanol?

As seen in previous paragraphs, exposure to ethanol either in chronic or acute way, during prenatal life or at adulthood leads to modification in the expression levels for both GluN2A and GluN2B subunits. Therefore in all examples given, we should anticipate some changes in the ratio between the expressions of these two critical subunits or the NMDA-Rs. Importantly, this ratio has been shown to govern the direction of synaptic plasticity in a given neuronal network [5]. Specifically, when GluN2A-to-GluN2B ratio is low, LTP is more likely to occur through the studied synapse rather than LTD and vice versa. Interestingly, different examples of specific behavioral alterations have been shown to be accompanied with modification of the GluN2A-to-GluN2B ratio. Recently, CD1 mice offspring from hypomagnesemic dams showed a decreased ratio while important behavior such as anxiety was highly expressed [89]. In a preclinical model of Alzheimer disease, it was shown that the intracellular pathway linked to the ratio was disturbed [90]. At clinical level, GluN2B-to-GluN2A ratio has been shown to increase in electrical status epilepticus in sleep and in refractory convulsive status epilepticus or in malformation of cortical development [91]. Increasing the ratio also accompanied fear memories that are resistant to retrieval-dependent memory destabilization [92]. Furthermore, GluN2A-to-GluN2B ratio appears as a common synaptic trait in rat and primate models of levodopa-induced dyskinesias as well as in dyskinetic Parkinson's disease patients [93]. Concerning ethanol, the effects of only two binges was shown to modify this ratio and hence synaptic plasticity in CA1 area of the dorsal hippocampus and consequently, learning capabilities [66], (Fig. 2). In the same vein, a lower ratio was obtained after pre- and postnatal ethanol exposure in rats [59]. Heterozygous knock-in mice for a mutant of the GluN1 subunit (F639) reducing ethanol inhibition of NMDA-Rs affected ethanol-induced behaviors in these mice. Interestingly these mice showed normal expression of both GluN1 and GluN2B but a small reduction of GluN2A expression notably in the mPFC [94]. These animals consumed less ethanol than the wild-type in daily limited-access sessions but consumed more in an intermittent 24 h access paradigm. In addition, many (but not all) effects following exposure to ethanol were significantly different from wild-type animals, suggesting that the GluN1 mutation and potentially the changes in GluN2A-to-GluN2B ratio are the key actor in these behaviors [94]. Ethanol exposure from PND 4–9 by gavage revealed changes in NMDA-Rs subunit expression in whole-cell lysate from dorsal hippocampus and in particular in the synaptic compartment where GluN2B subunit was selectively decreased, thus increasing the ratio [95]. Such changes have been shown to

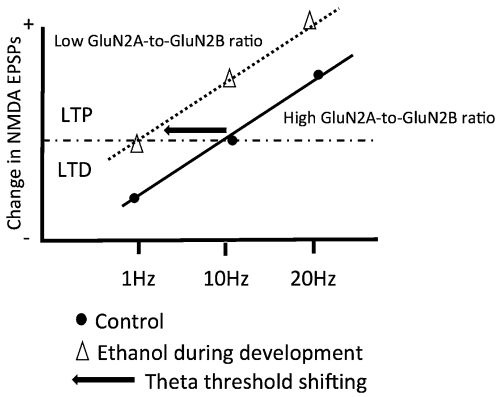

Fig. 2 Schematic representation of the changes in NMDA EPSPs as a function of frequency stimulation in hippocampus slice. At low frequency (1 Hz) LTD is triggered in control animals (black dots) whereas after ethanol LTD is abolished (open triangle; no LTP or LTD). At 10 Hz, there is no plasticity in control but an LTP is observed after ethanol. Finally, at high frequency the two populations show LTP but with different magnitudes. Comparing the stimulating frequency at which there is no plasticity reveals a leftward shift of this threshold (theta). Such shift is believed to be dependent on an overexpression of GluN2B subunit [5]. The scheme is adapted from results in Refs [59, 66] obtained in CA1 area of the hippocampus. However, a similar shift of the threshold for the expression of synaptic plasticity (i.e., LTP and LTD) can be obtained in other studies

be critical for trace fear conditioning, a form of Pavlovian conditioning [95]. Interestingly, interfering specifically with one NMDA-Rs subunit has been suggested to represent an intriguing therapeutic approach in dyskinesia patients [93] while manipulating the NMDA-Rs subunit ratio may both facilitates synaptic plasticity and memory but also could protect the synapse and its established plasticity to perhaps, save the specific trace [96].

Conclusion

The preclinical and clinical researches performed the last 50 years or so on the effects of ethanol exposure on CNS functioning have brought many information to better understand why and how the brain is getting jeopardize by this drug of abuse. Globally, it seems that ethanol exposure will in any cases increase NMDA-Rs function throughout exposure, an effect revealed during withdrawal characterized by a hyperexcitatory state. If the precise site of action of ethanol on the NMDA-Rs structure is not yet clearly identified, ethanol's effects occur through the modulation of NMDA-Rs subunits expression. This phenomenon is highly dependent on brain area but also depends on substructure levels and ultimately it happens that important alcohol-related behaviors are under the control of GluN2A-to-GluN2B expression ratio since this ratio will ultimately define the synapses

capacity to perform plasticity and then to control the flow of information through the dedicated network.

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