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Alcohol Modulation of Extra-synaptic Gamma-aminobutyric Acid Type A Receptors

Ayla Arslan

International University of Sarajevo, Faculty of Engineering and Natural Sciences, Department of Genetics and Bioengineering, Hrasnicka Cesta 15, Ilidža 71210 Sarajevo, Bosnia and Herzegovina

Abstract
Development of effective treatment agents for the alcohol use disorders requires the detailed understanding of molecular targets of alcohol in the brain. The gamma-aminobutyric acid type A receptors ($GABA_ARs$) are the major molecular targets of alcohol in the central nervous system. Mediating inhibitory neurotransmission upon GABA binding in the vertebrate central nervous system, $GABA_ARs$ are heteropentameric chloride channels, assembled from a large subunit pool encoded by 19 distinct genes. It is the subunit composition that determines the receptor's biophysical properties, neurotransmitter affinity, the pharmacology, and the position on the cell i.e., synaptic or extra-synaptic. This review paper briefly presents the alcohol modulation of a specific GABAAR subtype located at the extra-synaptic sites with a subunit composition of α, β and δ.

Keywords: Alcohol, GABA, extra-synaptic, GABA (A) receptors, δ (delta) subunit

1. Introduction

Affecting about 18 millions of adult Americans, alcohol abuse and alcohol dependence are classified as alcohol use disorders (AUD), which are not satisfactorily treatable. For example, Benzodiazepines (BZs), used for the treatment of symptoms of AUDs, cause addiction and Naltrexone, despite being an effective therapeutic agent, has severe side effects (Liang and Olsen, 2014). Thus, development of better treatment agents for the AUDs is essential which requires a detailed understanding of molecular targets of alcohol.

Accumulating evidence in the literature suggests that gamma-aminobutyric acid type A receptors ($GABA_ARs$) are the major target of alcohol in the brain (Mihic and Harris, 1997; Boehm et al., 2004; Kumar et al., 2009). $GABA_ARs$ are the member of "Cys-loop receptors" together with nicotinic acetylcholine receptors (nAChR), the 5- hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors, the zinc-activated ion channel (ZAC) and the glycine receptors (GlyR) (reviewed in Sine and Engel, 2006). They are GABA-gated heteropentameric chloride channels responsible for the fast inhibitory synaptic transmission in the vertebrate central nervous system (CNS) (reviewed by Sieghart and Sperk 2002). The $GABA_ARs$ display a rich molecular and cellular diversity, which result in distinct functional roles. Assembled from a large subunit pool, receptor subunit composition affects the receptor gating, kinetics and the response to allosteric modulators (Haas and Macdonald, 1999; Lavoie, et al., 1997). Besides, subunit composition is a determinant of the cellular and subcellular localization of the receptor, i.e., synaptic or extra-synaptic sites (Jones et al, 1997; Brickley et al., 2001; Goetz et al., 2007).

2. The Subunit Composition of $GABA_ARs$: Synaptic and Extra-synaptic Receptors

One of the most distinguishing features of $GABA_ARs$ is the wide repertoire of subunits from which the receptor assembles (Seeburg et al., 1990). The $GABA_ARs$ are assembled from a pool of 19 subunits ($\alpha1-\alpha6$, $\beta1-\beta3$, $\gamma1-\gamma3$, δ, ε, θ, π, $\rho1-\rho3$) (Rudolph and Mohler, 2006). The expression of the subunit genes is age- and region dependent (Wisden et al., 1992; Laurie et al., 1992a, b; Fritschy and Mohler, 1995; Schwarzer et al., 2001). The most abundant $GABA_ARs$ in the mammalian brain seem to be the combination of $\alpha\beta\gamma2$ subunits with a subunit ratio of $2\alpha/2\beta/1\gamma$ (Ernst et al., 2003). These $\gamma2$ containing $GABA_ARs$ ($\gamma2\text{-}GABA_ARs$) mediate classical fast synaptic inhibition (phasic inhibition) and massively clustered in the synapses. On the other hand δ subunit containing $GABA_ARs$ receptors ($\delta\text{-}GABA_ARs$), typically in combination with $\alpha6$ and β subunits in the cerebellum (Jones et al, 1997; Brickley et al., 2001); and in combination with $\alpha4$ and β subunits (Patel et al., 2014) in the forebrain, are located extra-synaptically or perisynaptically (Nusser et al., 1998; Wei et al., 2003). Activated by GABA diffusing out of the synaptic cleft, these receptors mediate a special form of inhibition called the tonic inhibition characterized by a higher affinity for GABA and with a slower

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desensitization rate (Nusser and Mody, 2002). Tonic inhibition is critical for the threshold for the action potential generation. It shunts the excitatory synaptic signals controlling neuronal excitability (Hamann, et al., 2002, Semyanov et al., 2004). Thus the extra-synaptic $GABA_A$Rs mediate a physiologically different form of GABAergic signaling than the synaptic receptors (Brickley and Mody 2012). The diversity of $GABA_A$R subunits with distinct physiological functions (Mody and Pearce, 2004) is also apparent at the level of its ligands. Benzodiazepines, barbiturates, alcohol and neurosteoids are the modulators of $GABA_A$Rs with differences in efficacy, potency and binding sites in a subunit dependent manner (Goetz et al, 2007). In this study we will present the alcohol modulation of extra-synaptic receptors containing $\alpha4\beta\delta$ or $\alpha6\beta\delta$ subunits (δ-$GABA_A$Rs).

3. The structure of the $GABA_A$Rs

Until last year, the molecular structure of a $GABA_A$R subunit complex was based on the studies of the muscle nAChR from the electric organ of the torpedo ray and the snail acetylcholine receptor binding protein (AChBP) (Brejc et al., 2001; Cromer et al., 2002; Ernst et al., 2003; Unwin, 2003, 2005; Sine and Engel, 2006). However, the recent work done by Miller and Aricescu (2014) reports the crystallized structure of homomeric $\beta3$ subunit containing $GABA_A$Rs ($GABA_A$R-$\beta3$cryst) at 3Å resolution which provides a direct overview for the receptor structure for the first time. Together with the recent structural data from $5HT_3$ receptors (Hassaine et al., 2014), these studies confirm the characteristics of eukaryotic Cys-loop receptors (reviewed by Lynagh and Pless, 2014).

$GABA_A$Rs are pentamers consist of five subunits arranged counterclockwise (i.e., $\gamma, \alpha, \beta,$) around a central pore. Each subunit comprises a long N- terminus located at the extracellular domain (ECD), followed by four transmembrane domains (TM1–TM4), and a short extracellular C-terminal. There is a large intracellular loop between the third and fourth transmembrane domains. According to Miller and Aricescu (2014) the receptor looks like a cylinder with a height of 110Å and with a diameter of 60 to 80Å. From the extracellular side, the receptor is surrounded by 15 N-linked glycans. Each extracellular domain (ECD) comprises an amino-terminal α-helix ($\alpha1$) followed by ten β–strands in parallel with the structure of other family members (reviewed in Lynagh and Pless, 2014). A second α–helix ($\alpha2$), between β-strands 3 and 4, is located under the $\alpha1$ helix. The pentameric transmembrane domain (TMD) is composed of four additional helices (M1–M4) from each subunit that come together to form a lining a pore with M2 segments. The subunit assembly is mediated by hydrogen bonds, van der Waals forces and salt bridges in the subunit ECDs, which also involve the neurotransmitter binding pocket.

When subunits are assembled in to the heteropentameric receptor, the neurotransmitter binding pocket, i.e., the GABA binding site, is located at the interface between N terminus extracellular domains of the β and α subunits, which constitutes a "principal face" and a "complementary face", respectively. As reported by Miller and Aricescu (2014), the principal face of human $GABA_A$R involves the $\beta4$ strand and residues Asp95-Leu99, Glu155-Tyr159, Phe200-Tyr205 in the β subunit. The complementary face corresponds to the residues Tyr62-Gln64 and Leu125-Arg129 in the α subunit. On the other hand, agonist sensitivity seems to be affected also by the motifs, which are not located in the neurotransmitter binding pocket (Korpi and Luddens, 1993; Böhme et al., 2004). For example a domain (S238-V264) in the δ subunit might be important for the high agonist affinity of the extra-synaptic $\alpha4\beta3\delta$ receptors (You and Dunn, 2007) compared to synaptic $\gamma2$ containing receptors. Thus, following the formation of receptor and agonist complex at the neurotransmitter binding site, the agonist affinity and efficacy might be affected by all subunits (see Unwin 2005).

4. Alcohol modulation $GABA_A$Rs

Alcohol has profound effects in the brain. Interacting with multiple neurotransmitter systems (Valenzuela CF., 1997), its impact is characterized by intoxicating, sedative, anxiolytic and addictive features in the behavioral level (Bayard et al., 2004). Ethanol affects many ion channels, including the NMDA glutamate receptors (Hanchar et al., 2005). In the CNS, $GABA_A$Rs are the major targets of alcohol (Mihic and Harris, 1997; Boehm et al., 2004; Hanchar et al., 2005; Kumar et al., 2009). In addition to direct allosteric effect of ethanol on $GABA_A$Rs (Deitrich et al., 1989), there are also indirect effects on the receptor due to ethanol mediated increase in the levels of presynaptic release of GABA (Yang et al., 2000; Roberto et al., 2003; Ming et al., 2006; Theile et al., 2008; Mameli et al., 2008) and neuroactive steroids (Caldeira et al., 2004; Mameli and Valenzula 2006; Izumi et al., 2007), which are the modulators of GABA receptors. Besides, ethanol affects the phosphorylation of $GABA_A$Rs, which in turn lead to an increase in the GABA sensitivity (Hodge et al., 1999, 2002; Kumar S., 2009). For allosteric effects, ethanol sensitivity depends on the $GABA_A$R subtypes. In general $\gamma2$- $GABA_A$R subtypes are sensitive to ethanol at doses attained by severe intoxication (Kumar S., 2009). The extra-synaptic δ–GABAARs are thought to be most

sensitive to ethanol, which will be discussed in the following section.

5. Alcohol and extra-synaptic $GABA_ARs$

In general, 1-3 mM blood ethanol levels can result from drinking half a glass of wine or less (Goetz et al 2007). This is especially important as the extra-synaptic δ–$GABA_ARs$ are thought to be most sensitive to ethanol at levels of social drinking, i.e., less than 30 mM (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003, 2006; Wei et al., 2003; Hanchar et al., 2005; 2006; Santhakumar et. al., 2007; Glykys et al., 2007; Mody et al., 2007; Olsen et al., 2007). Studies of δ subunit knock out mice have shown the impact of extra-synaptic δ - $GABA_ARs$ for mediating the effects of ethanol (Mihalek et al., 2001). These mice show less responsiveness to the anticonvulsant effects of ethanol, a decreased response of excitability to ethanol withdrawal, and a less preference for ethanol compared to wild-type mice. This phenomenon is dependent on β subunit with β3 isoform providing maximal sensitivity to ethanol (Wallner et al., 2003).

A direct evidence of the effect of ethanol via δ-$GABA_ARs$ on cerebellar granule cells has been shown by the R100Q mutation in the α6 subunits of the alcohol non-tolerant rats. Cerebellar granule cells express the extra-synaptic receptors with a specific partnership of α6 and δ subunits together with the β subunit (Jones et al., 1997; Brickley et al., 2001). Rats homozygous for the mutation (α6-100QQ) have an increased alcohol-induced ataxia and they have an increased activity of α6βδ receptors enhanced by alcohol in cerebellar granule cells (Hanchar et al., 2005, 2006). On the other hand α6 knock-out mice do not show any distortion of alcohol sensitivity (Homanics et al., 1997). However, this may result from the adaptive responses of the cerebellar granule cells to the absence of α6 subunits, which could mask the relation of corresponding receptors (α6 and β subunits which has a specific partnership with δ subunit in the cerebellum) and ethanol actions. Indeed, the α6 subunit knock-out mice have increased expression of TASK-1 channel in these cells, which may impact on ethanol sensitivity. In line with this, TASK-1 knock-out mice are more sensitive to ethanol in behavioral level, which might explain the unchanged ethanol sensitivities of α6 knockout mice of $GABA_AR$ (reviewed by Korpi et al., 2007). Other experiments done with the recombinant expression systems have shown that 3-30 mM alcohol is enough to activate δα4β and δα6β subunit containing receptors (Wallner et al., 2003; 2006; Sundstrom-Poromaa et al., 2002). This effect is shown to increase the tonic inhibition (Wei et al., 2003; Hanchar et al., 2005; Glykys et al., 2007; Santhakumar et al., 2007; Liang et al., 2008), the specific form of inhibition mediated by the δ-$GABA_ARs$.

As a result, several lines of evidences from the studies of recombinant expression systems and electrophysiological recordings converge on the hypothesis that physiologically relevant, low dose (less than 50 mM) actions of ethanol is mediated by extra-synaptic δ- $GABA_ARs$. Thus, a glass of wine activating the extra-synaptic receptors could potentiate GABAergic tonic inhibition in the striatum and cerebellum via α4β3δ and α6βδ receptors respectively (Hanchar et al., 2005, 2006; Olsen et al., 2007).

On the other hand, these results generated some controversy in the field as the findings regarding the high alcohol affinity of the extra-synaptic receptors have not been replicated by some groups and presynaptic mechanisms have been proposed for the alcohol potentiation of GABAergic system (Carta et al., 2004; Borghese et al., 2006; Botta et al., 2007a, b; Korpi et al., 2007; Baur 2009). Several experimental errors or methodological issues may cause this contradiction. For example, in one of the studies who fail to replicate the physiologically relevant alcohol potentiation of extra-synaptic receptors (Botta et al., 2007), the magnitude of GABAergic tonic currents has been described as 55 pA, which is much higher than tonic currents (i.e., less than 30 pA) described in many other studies for the comparative age and cell types of rodents (cited in Otis, 2008). δ subunit is a rare isoform of GABA receptor subunits by means of its distribution in the brain: Its expression is restricted to cerebellar granule cells (Jones et al., 1997), dentate gyrus granule cells in the hippocampus (Sun et al., 2004) and ventrobasal nucleus of the thalamus and neocortex (Cope et al., 2005; Glykys et al., 2007). Thus, it is reasonable to expect some experimental caveats for the *in vitro* ectopic expression of δ- $GABA_ARs$ (Arslan et al., 2014). For example, in vitro expression of recombinant δ subunit is generated variable results by means of clustering on the cell membrane. Regarding this, one study reported that *in vitro* expression of recombinant δ subunit shows a diffusely distributed pattern on the cell membrane but *in vivo* studies show that δ subunit containing receptors form clusters (Sun et al., 2004). In parallel with the latter finding, Arslan et al., (2014) reported that recombinant δ subunit when expressed in the primary cultures of hippocampal neurons gave a punctate immunostaining on non-permabilized cells. Here it is important to consider many factors that could contribute to this discrepancy. For example Arslan et al. (2014) used N-terminus GFP tagged version of δ subunit where as Christie et al (2006) used the

C-terminus GFP tagged version of this subunit. Also, low *in vitro* expression profile of recombinant δ subunit (Arslan et al., 2014) and its restricted ability to form functional receptors *in vitro* may produce experimental failures (Olsen et al., 2007; Santhakumar et al., 2007; Otis, 2008). Moreover there are some possible effect of species differences in alcohol and alcohol antagonist responses (Wallner, et al., 2014). For example, a recent study suggests that there are some significant differences in the pharmacology of murine and human α4β1δ receptors (Villumsen et al., 2015). Therefore, it is clear that methodological issues should be carefully considered for studies with δ- $GABA_A$Rs in general and for the effects of alcohol on these receptors in particular.

6. Alcohol binding site

Perhaps a direct evidence for the ethanol enhancement of δ-$GABA_A$Rs would come from studies showing the alcohol binding site on the δ-$GABA_A$Rs. For the synaptic receptors like α1β2γ2 containing ones, mutagenesis and labeling studies have led to the identification of several amino acid residues in transmembrane domain critical for alcohol modulation. For example, site directed mutagenesis studies identified S270 and A291 on the second and third transmembrane domain of α subunit of $GABA_A$Rs critical for allosteric modulation by alcohol (and volatile anesthetics) (Mihic et al., 1997). Many of these residues are involved in the enhancement of receptor function by alcohol as positive allosteric modulator (Mihic et al., 1997; Jenkins et al., 2001; 2002; McCracken et al., 2010).

Regarding extra-synaptic δ-$GABA_A$Rs several studies have reported that a competitive antagonist of ethanol, Ro15-4513, prevents many of the behavioral aspects of ethanol intoxication (Suzdak et al., 1986; Lüddens et al., 1990; Hanchar et al., 2006, Wallner et al., 2006). Experiments utilizing the radiolabeled Ro15-4513 have shown that ethanol can displace Ro15-4513 on the δ subunit (Hanchar et al., 2006, Wallner et al., 2006). Addressing this, a new extracellular alcohol/imidazobenzodiazepine (Ro15-4513) site has been identified for the δ-$GABA_A$Rs (Wallner et al., 2014). By the use of site directed mutagenesis experiments and homology modeling, Wallner et al. (2014) have shown that this site, involving the residue Y76 in the β3 subunit, is located at the interface between the α4/α6 and β3 subunit of δ-$GABA_A$Rs and matches with the residue (γ2 T81) of benzodiazepine site of γ2 - $GABA_A$Rs. Thus the binding site of ethanol is likely located at a site on extra-synaptic δ-$GABA_A$Rs corresponding to benzodiazepine site of synaptic γ2 - $GABA_A$Rs.

7. Conclusion

The current pharmacotherapy for AUDs is not effective satisfactorily (Liang and Olsen 2014). Development of better treatment agents for the AUDs requires the detailed understanding of molecular targets of alcohol relevant to its effects in the brain. Accumulating evidences from the studies of recombinant expression systems, electrophysiological recordings from the neurons and labeling experiments converge on the hypothesis that physiologically relevant, low dose actions of ethanol is mediated by extra-synaptic δ-$GABA_A$Rs. This action is likely to occur by an allosteric mechanism corresponding to a BZ site in the ECD of the δ- $GABA_A$Rs (Wallner et al., 2014). But the ethanol action on $GABA_A$Rs does not seem to be limited to one site. Probably there are multiple sites, some of which are physiologically critical while others not (Mihic et al., 1997; Jenkins et al., 2001; 2002; McCracken et al., 2010; Wallner et al., 2014). Current developments in our understanding of the structure of $GABA_A$Rs (Miller and Aricescu, 2014) and related proteins from eukaryotic and prokaryotic organisms (reviewed by Lynagh and Pless, 2014) will likely answer these questions and initiate new opportunities addressing the alcohol actions on $GABA_A$Rs. Based on the available X-ray data, molecular dynamics (MD) simulations have the potential to offer an atomic level dynamics of the conformational changes on the receptor during the process of signal transmission, and the effect of allosteric modulators on this process. These opportunities will not only lead to the verification of present data and clarification of inconsistencies in the literature described so far but also elucidation of allosteric interactions of ethanol besides to other $GABA_A$R modulators at level of atomic scales for better perspectives of drug design. Thus, a significant progress is expected in the field to address the mechanism of allosteric modulation of various ligands besides to ethanol on different subtypes of $GABA_A$Rs, including extra-synaptic δ-$GABA_A$Rs.
subtypes.

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