The Role of Beta Subunit Variant on the Properties of Delta-Containing Extrasynaptic $GABA_A$ Receptors

Master's thesis
University of Turku
Faculty of Medicine
MSc Degree Programme in
Drug Discovery and Development
June 2017

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BENKHEROUF, ALI: The Role of Beta Subunit Variant on the Properties of Delta-Containing Extrasynaptic $GABA_A$ Receptors.
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Muscimol, a psychoactive constituent of Amanita muscaria, activates all $GABA_A$ receptor subtypes with exceptionally high affinity to αβδ. This is explained by δ subunit role on muscimol extremely slow dissociation from these receptors. However, the contributions of $GABA_AR$ β subunit variant to the affinity state of αβδ receptors and function were not described in similar detail as α subunit variant in the literature. The extrasynaptic δ-containing receptors are in a great deal responsible for the so-called tonic inhibition in the brain.

This study aims to understand the role of $\beta_{1,2,3}$ subunits in muscimol binding to δ-containing extrasynaptic $GABA_A$ receptors. We performed transient expression of recombinant $GABA_AR$ subtypes in HEK293 cells and determined the association and dissociation rates of [^{3}H]muscimol binding to recombinant α6β1δ, α6β2δ and α6β3δ receptors using radiolabeled binding kinetics approaches.

α6$\beta_{1,2,3}$δ recombinant receptors showed distinct binding kinetics. In α6β1δ and α6β2δ recombinant receptors, [^{3}H]muscimol association was similar and significantly slower than α6β3δ. Moreover, dissociation of the binding was very slow especially to α6β2δ recombinant receptor compared to α6β1δ and α6β3δ that were similar. The remarkable slow dissociation of [^{3}H]muscimol from all three receptor subtypes confirms the studies indicating α6β_{1-3}δ high affinity state being independent of β subunit.

Further electrophysiological studies are needed to reveal how different β subunits affect binding site of αβδ $GABA_A$ receptors in terms of ligand sensitivity and efficacy. This leads to an improved pharmacological characterization of extrasynaptic αβδ receptors and possible development of selective allosteric modulators with therapeutic significance for alcoholism, anesthesia and epilepsy.

Keywords: GABA-A receptors, muscimol, binding, association, dissociation, affinity

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1. INTRODUCTION

1.1 $GABA_A$ receptors

γ-Aminobutyric acid type A receptors ($GABA_AR$) are key sites in rapid synaptic inhibition in vertebrate brain (Olsen & Sieghart, 2008). $GABA_ARs$ contain an intrinsic chloride channel that is opened by the binding of GABA. The entrance of chloride ions into mature neurons leads to hyperpolarization and/or stabilization of the membrane potential and thus inhibition of the neuron (Macdonald & Olsen, 1994). The modulation of receptor function occurs through allosteric binding sites by different clinically significant drugs, such as benzodiazepines, steroids, anesthetics and barbiturates (Sieghart, 1995) which indicates the central role of $GABA_ARs$ as targets for modulation of CNS excitability.

1.2 Molecular and pharmacological subtypes of $GABA_ARs$

Molecular cloning has identified 19 mammalian genes that code for $GABA_AR$ hetero-oligomeric protein subunits which belong to 8 subunit classes: α1-α6, β1-β3, γ1-γ3, δ, ε, π, θ and ρ1-ρ3 (Olsen & Sieghart, 2008). Brain regional subunit mRNA expression is heterogeneous with cell-type specificity (Laurie et al., 1992; Wisden et al., 1992). This produces receptor subtypes with distinctive functional and pharmacological properties. Differential distribution of receptor subtypes in the CNS enables development of drugs that act on a receptor subtype with restricted regional localization in the brain. With subtype-selective drugs it may be possible to modulate specific physiological functions without undesirable side effects.

Most $GABA_ARs$ are pentameric complexes that contain α, β and γ subunits where subunit stoichiometry is 2α:2β:1γ (Figure 1) (Tretter et al., 1997). The γ subunit is needed for binding of classical benzodiazepines as γ2 subunit is considered as the γ isoform present in over 90% of αβγ receptors (Pritchett et al., 1989; Wisden et al., 1992; McKernan & Whiting, 1996). γ2 associates with all α subunits, the subunit class mainly determining benzodiazepine pharmacology (Sieghart, 1995). Studies utilizing $GABA_AR$ knock-in mouse lines

with a point mutation rendering the respective α subunit benzodiazepine-insensitive have indicated the mediation of benzodiazepine-induced sedation and amnesia by receptors containing α1 and anxiolysis by receptors containing α2, respectively (Möhler et al., 2002). Receptors that do not contain a γ subunit are generally regarded insensitive to benzodiazepines (Pritchett et al., 1989).

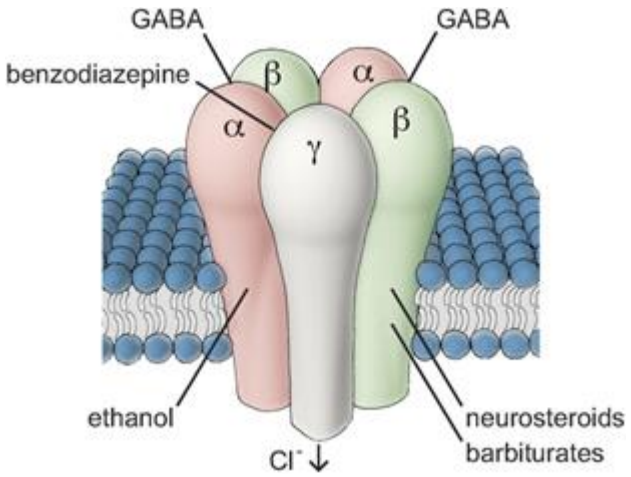

Fig. 1. $GABA_A$ receptor subunit composition (Uusi-Oukari and Korpi, 2010)

1.3 Synaptic $GABA_A$Rs mediate phasic inhibition

Phasic inhibition is produced by inhibitory postsynaptic currents (IPSCs) that emerge from synaptic contacts during synaptic release of inhibitory transmitter which in turn transiently inhibits neurons for 10-100 milliseconds (Semyanov et al., 2004).This synaptic transmission is mediated by $GABA_A$Rs clustered in postsynaptic densities. Postsynaptic clustering of $GABA_A$ receptor subtypes α2βγ2 and α3βγ2 is induced by interaction of α2 and α3 subunits with gephyrin (Wu et al., 2012), a multifunctional protein that serves as a sub-synaptic scaffold organizing the spatial distribution of receptors and other proteins in inhibitory postsynaptic membranes. The interaction of $\alpha_{1\text{-}3}$βγ2 $GABA_A$ receptors with the postsynaptic cytoskeleton is regulated by the activity-dependent and calcineurin-regulated phosphorylation state of γ2 subunit. Transmembrane domain 4 and intracellular domain of the γ2 subunit has been shown to be essential for recruiting gephyrin to the synapse. The role of gephyrin is to stabilize clustered $GABA_A$Rs at the cell surface (Vithlani et al., 2011).

Therefore, synaptic $GABA_A$Rs are of αβγ-type receptors where the classic benzodiazepines predominantly augment the synaptic (phasic) inhibition.

1.4 Extrasynaptic $GABA_A$Rs mediate tonic inhibition

The continuous activation of extrasynaptic $GABA_A$Rs by ambient GABA results in tonic inhibition. Extrasynaptic GABA originates when GABA escapes from synaptic cleft (GABA spill-over) (Brickley et al., 1996; Mitchell & Silver, 2000; Semyanov et al., 2003) or when GABA is released through unconventional mechanism (possibly non-vesicular) from neurons and glia (Wang et al., 2002; Demarque et al., 2002; Lee et al., 2010). Extrasynaptic receptor subtypes mediating tonic inhibition mainly include δ subunit-containing receptors (Nusser et al., 1998; Nusser & Mody, 2002; Stell & Mody, 2002). They possess characteristics needed for tonic activity since they have very high GABA-affinity and hence get activated by low GABA concentrations. Moreover, they do not desensitize upon a prolonged presence of GABA (Saxena & Macdonald, 1996) thus being able to maintain continuous inhibition.

1.5 Expression of δ-containing $GABA_A$Rs in the CNS

γ2 and δ subunits are assembled into separate receptors, and δ is present only in α6/4β-containing extrasynaptic combination (Quirk et al., 1995; Jechlinger et al., 1998; Pöltl et al., 2003). A significant group of $GABA_A$Rs especially in cerebellar granule cells (CGC) are those containing the δ subunit (Quirk et al., 1995; Sur et al., 1999). From the second postnatal week CGCs express six main $GABA_A$R subunit isoforms throughout the adulthood: α1, α6, β2, β3, γ2 and δ (Laurie et al., 1992; Jechlinger et al., 1998; Pöltl et al., 2003). Brain regional expression of α6 is restricted to CGCs, while α1, β2, β3 and γ2 are expressed highly in most of the brain regions (Lüddens et al., 1990; Laurie et al., 1992). Combinations α1βγ2, α6βγ2 and α6βδ have been shown to be the major receptor subtypes in CGCs (Jechlinger et al., 1998; Pöltl et al., 2003). In hippocampus δ subunit is expressed mainly in dentate granule cells (DGC) (Wisden et al., 1992). In these cells δ combines with α4 and β subunits to form α4βδ receptors (Sur et al., 1999). These receptors have been implicated in mediating tonic inhibition in DGCs (Overstreet & Westbrook, 2001; Nusser &

Mody, 2002; Stell & Mody, 2002). In addition to CGCs and DGCs, δ is highly expressed in thalamus and in moderate levels in cerebral cortex and in nucleus accumbens (NAc) (Wisden et al., 1992).

1.6 Up-regulation of extrasynaptic δ-containing receptors and brain function

Delta subunit expression is drastically up-regulated in rodent neurons *in vitro* by various types of glutamatergic depolarization (Gault & Siegel., 1998; Martikainen et al., 2004; Salonen et al., 2006; Uusi-Oukari et al., 2010). At present it is not known whether this up-regulation of $GABA_AR$ subunit mediating tonic inhibition occurs in living brain during prolonged excitation. However, in stargazer mutant mice that lack functional stargazing, a protein regulating synaptic targeting and anchoring of AMPARs (Letts, 2005; Osten & Stern-Bach, 2006), extrasynaptic α4βδ and α6βδ $GABA_ARs$ are drastically down-regulated (Payne et al., 2006; Payne et al., 2007). This suggests that AMPAR activity up-regulates αβδ receptor expression also *in vivo*. Significant alterations occur in brain glutamate levels and/or in glutamate receptors sensitivity during epileptic seizures, cerebrovascular ischaemia and alcohol withdrawal (Chapman, 1998; Dirnagl et al., 1999; Dodd et al., 2000). This suggests that up-regulation of $GABA_AR$ δ subunit may be a mechanism to reduce overexcitation in these conditions. This hypothesis is supported by the recent findings that tonic inhibition mediated by δ-containing $GABA_ARs$ was strongly increased in DGGCs after traumatic brain injury (Mtchedlishvili et al., 2010; Kharlamov et al., 2011) and in pilocarpine-induced status epilepticus (Zhan & Nadler, 2009). The important role of α4βδ receptors in neuroprotection was demonstrated in striatal slices where muscimol efficiently protected neurons against quinolinic acid-induced excitotoxic insults in wild-type but not in δKO mice slices (Santhakumar et al., 2010).

1.7 The role of β subunit variant in $GABA_A$ Receptors

In native $GABA_AR$, β1 is known to be a rare subunit expressed mainly in cortex, hypothalamus and hippocampus. β2 and β3 equally represent almost 50% of all β subunits and are remarkably expressed in hippocampus, cerebellum,

striatum, cortex and olfactory bulb. β2 is also highly expressed in thalamus (Lee and Maguire, 2014). The contribution of $GABA_AR$ β subunit variant to ligand affinity and receptor function has not been studied as extensively as α subunit variant. One reason could be the limitation of allosteric modulators binding selectivity to β1, β2, or β3 subunits. Nevertheless, Thompson et al., 2004 reported on salicylidene salicylhydrazide, a negative allosteric modulator which selectively inhibits β1 containing $GABA_ARs$ with high potency. Moreover, several genetic models were produced to understand the role of beta subunits in animal behavior. Mouse models with β2 knockout displayed elevation in locomotor activity and decreased response to benzodiazepines and GABA (Sur et al., 2001). β3 knockouts are used as mouse model for Angelman syndrome. They exhibit extensive loss of $GABA_ARs$ in CNS and only 10% survive within the first day. Increased activity, excitability, seizures and deterioration in motor performance were reported (Resnick et al., 1999; Krasowski et al., 1998; Homanics et al., 1997a; DeLorey et al., 1998). On the other hand, animal models for β1 knockouts are currently not available.

1.8 Muscimol

Muscimol is the principal psychoactive constituent of Amanita muscaria and related species of mushroom broadly used as a lead compound for drug development (Krogsgaard-Larsen et al., 1981). It is a conformationally restricted $GABA_A$-analog and agonist that activates all $GABA_AR$ subtypes (Krogsgaard-Larsen et al., 1979; Ogurusu et al., 1999). However, it acts as a superagonist with exceptionally high affinity to αβδ subtypes (Storustovu & Ebert, 2006; Quirck et al., 1995). The moderate superagonist behavior of muscimol on αβδ receptors is caused by reduced desensitization of αβδ receptors (Mortensen, 2010).

An earlier study revealed the slow association and dissociation rates of [^{3}H]muscimol binding to recombinant α6β2δ $GABA_A$ receptors (Taina, 2013). The association rate constant for α6β2δ subtype was 3.8-4.4-fold lower compared to α6β2γ2 recombinant receptors, but the difference didn't reach statistical significance. Dissociation of [^{3}H]muscimol was very slow from α6β2δ subtype, significantly slower than dissociation from the corresponding α6β2γ2

subtype. Hence, the high affinity of muscimol to αβδ receptors is suggested to be due to its extremely slow dissociation from these receptors.

Whether endogenous GABA shows similar binding kinetics as [^{3}H]muscimol on α6β2δ and α6β2γ2 recombinant receptors needs to be investigated. Moreover, using radiolabeled binding kinetics approaches, the association and dissociation rates of [^{3}H]muscimol binding to recombinant α6β1δ, α6β2δ and α6β3δ $GABA_A$ receptors are measured in this study. This gives a better understanding on the role of β1, β2, β3 subunits in muscimol binding to delta-containing extrasynaptic $GABA_A$ receptors.

2. RESULTS

2.1 Association of [^{3}H]GABA to recombinant $GABA_A$R subtypes

Results showed that the association of [^{3}H]GABA at room temperature to α6β2δ recombinant receptors was slower compared to α6β2γ2 (Figure 2). Two-way ANOVA analysis revealed a significant receptor subunits effect on [^{3}H]GABA association, $F(1,24) = 10.04$, $MSE = 47.2$, $p < 0.05$ (Bonferroni post hoc test) indicated that the association to α6β2δ was significantly slower than α6β2γ2 at 1 and 2 min time points ($p < 0.05$). An independent-sample *t*-test showed that there was a significant difference in [^{3}H]GABA association rate constants (k_{on}) in between the receptor subtypes $t(4) = 4.315$, $p < 0.05$, two-tailed. The association rate constant in α6β2δ subtype was 5.6 lower as compared to α6β2γ2 (Table1). The overall goodness of fit for the association curves were $R^2 = 0.55$ and 0.19 for α6β2δ and α6β2γ2 respectively.

Table 1: Association (k_{on}) rate constant of [^{3}H]GABA binding at room temperature in recombinant receptors expressed in HEK293 cells.

Recombinant Receptor	**k_{on} (M^{-1} x min^{-1})**	**± SEM**
α6β2**δ**	7.42 x 10^7	1.08 x 10^7
α6β2**γ2**	41.4 x 10^7	7.80 x 10^7

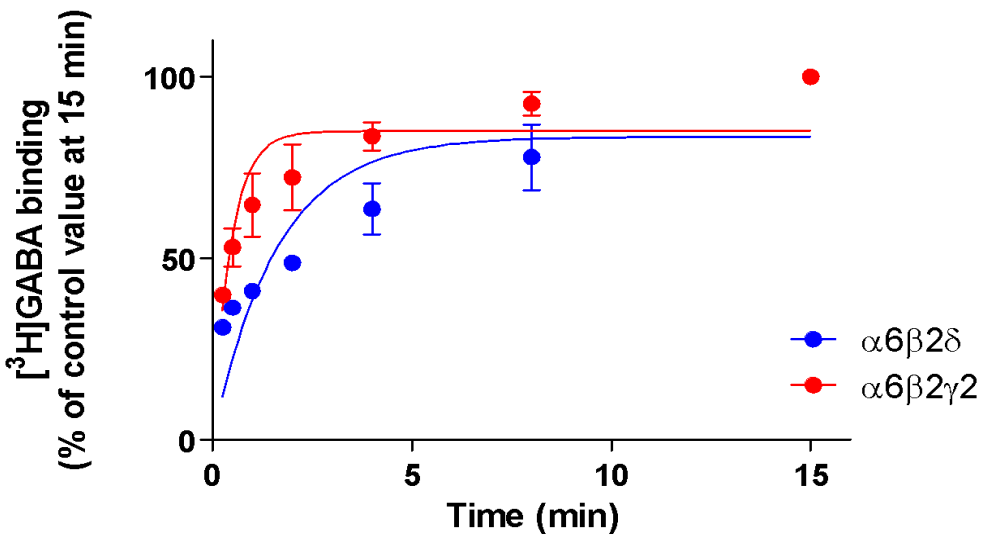

Fig. 2. Association of [^{3}H]GABA binding at room temperature to recombinant α6β2δ and α6β2γ2 receptors expressed in HEK293 cells (mean ± SEM; n=3).

2.2 Dissociation of [^{3}H]GABA from recombinant GABA$_A$R subtypes

The dissociation of [^{3}H]GABA from α6β2δ was very slow, significantly slower than α6β2γ2 subtype (Figure 3). Hence, the receptor subunit combinations significantly affected the dissociation (receptor subtype effect: $F(1,12) = 101.2$, $MSE = 46.05$, $p < 0.0001$; two way ANOVA). As a follow up test, Bonferroni post hoc analysis indicated that dissociation from α6β2δ was significantly slower than α6β2γ2 at 30 s, 60 s ($p < 0.0001$) and 3 min. ($p < 0.05$). Results also showed that 50% of [^{3}H]GABA was unbound from α6β2δ receptor at 99 s compared to α6β2γ2 receptor from which 50% was unbound at 19.2 s. At 30 min of dissociation, there was still [^{3}H]GABA remained bound to the recombinant receptors, 22% to α6β2δ and 7% to α6β2γ2 (Fig. 4). Furthermore, the dissociation (k_{off}) rate constant in α6β2δ was significantly lower than α6β2γ2, $t(4)= 5.666$, $p < 0.001$, Unpaired t-test, two-tailed (Fig. 4).

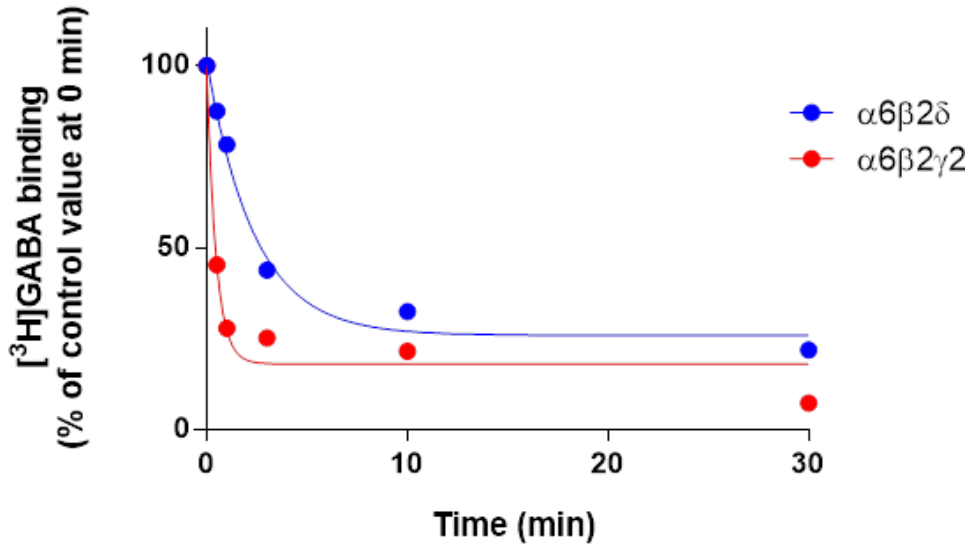

Fig. 3. Dissociation of [^{3}H]GABA binding at room temperature from α6β2γ2 and α6β2δ receptors. The dissociation from the δ containing receptor subtype (α6β2δ) was slower (k_{off} = 0.42 ± 0.07 min^{-1}) compared to α6β2γ2 (k_{off} = 2.16 ± 0.23 min^{-1}) (mean ± SEM; n=3).

2.3 Association of [^{3}H]muscimol to recombinant GABA$_A$R subtypes

The binding kinetics measurements of [^{3}H]muscimol at room temperature showed slow association especially for α6β1δ and α6β2δ compared to α6β3δ recombinant receptors (Figure 4). Nevertheless, at least 90% of maximal [^{3}H]muscimol binding level was reached to each of the three receptor subtypes at 8 min. There was a significant subunit effect observed on association (receptor subtype effect: $F(2,36) = 9.53$, MSE = 23.8, $p < 0.05$; two-way ANOVA). Post Hoc analysis using Bonferroni test revealed that [^{3}H]muscimol association to α6β1δ and α6β2δ receptors was significantly slower than α6β3δ, $p < 0.05$. However, the association to α6β1δ and α6β2δ subtype receptors was not significantly different. From Table 2, we note that [^{3}H]muscimol association (k_{on}) rate constant in α6β3δ was the highest. The association rate constants in α6β1δ and α6β2δ were significantly lower than α6β3δ ($p < 0.001$, one-way ANOVA, Tukey follow-up test). The difference in k_{on} between α6β2δ and α6β1δ did not reach statistical significance.

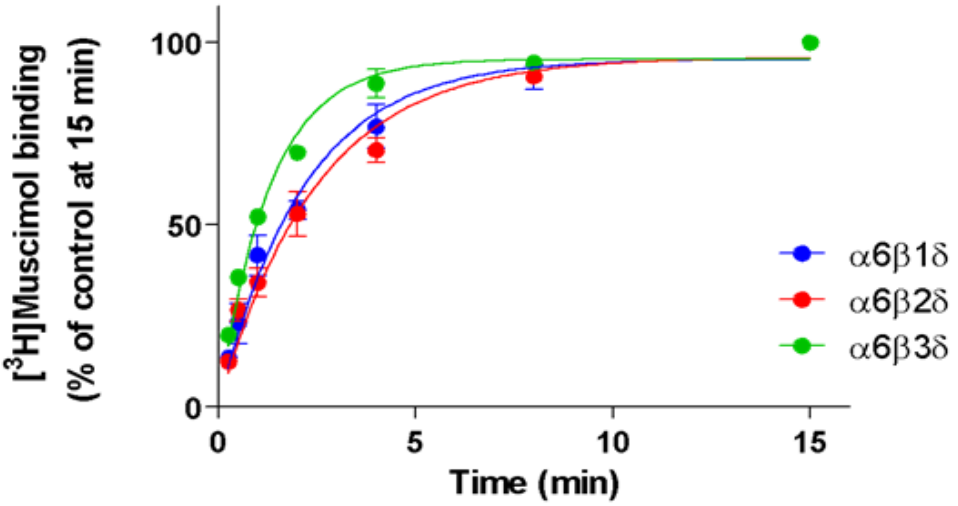

Fig. 4. Association of [^{3}H]muscimol binding at room temperature to recombinant α6β1δ, α6β2δ and α6β3δ receptors expressed in HEK293 cells (mean ± SEM; n=3).

2.4 Dissociation of [^{3}H]muscimol from recombinant GABA$_A$R subtypes

The dissociation of [^{3}H]muscimol from α6β1δ, α6β3δ and especially α6β2δ was very slow (Figure 5). A two-way ANOVA analysis yielded a significant effect for receptor subunit on dissociation, $F(2,40) = 5.72$, $MSE = 55.9$, $p < 0.05$. Bonferroni post hoc test showed that dissociation from α6β1δ and α6β3δ subtype receptors was not significantly different. However, [^{3}H]muscimol dissociation from α6β2δ was significantly slower than α6β1δ and α6β3δ, $p < 0.05$. The dissociation curve (Fig. 5) indicated that 50% of [^{3}H]muscimol was unbound from α6β2δ receptor at 11 min compared to α6β1δ and α6β3δ (4-5 min). Moreover, 31% of [^{3}H]muscimol remained bound to α6β2δ at 30 min of dissociation. The results indicated that [^{3}H]muscimol dissociation (k_{off}) rate constant in α6β2δ was the lowest compared α6β1δ and α6β3δ (Table 2). Dissociation rate constant difference was statistically significant for α6β2δ vs. α6β1δ, $p < 0.01$ and α6β2δ vs. α6β3δ, $p < 0.01$, but not significant for α6β1δ vs. α6β3δ (one-way ANOVA, Tukey follow-up test).

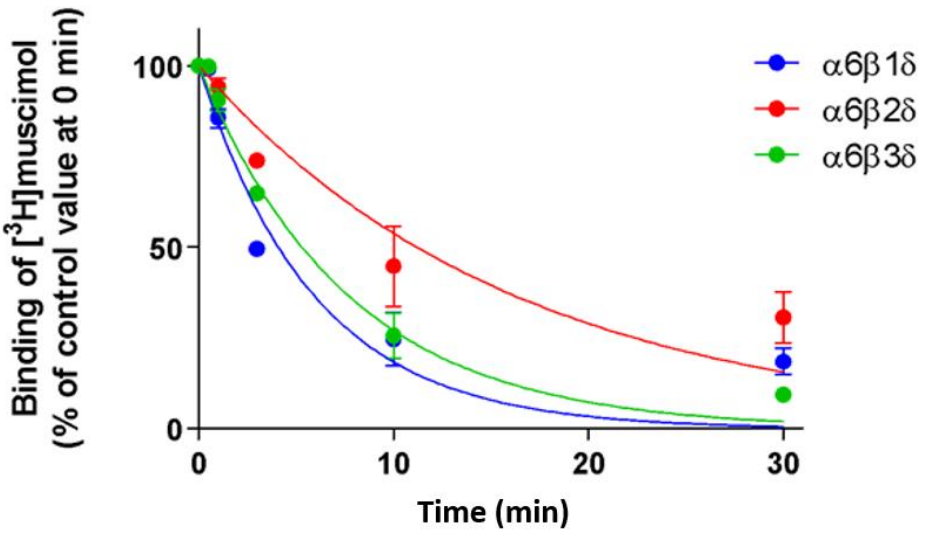

Fig. 5. Dissociation of [^{3}H]muscimol binding at room temperature from α6β1δ (n=3), α6β2δ (n=4) and α6β3δ (n=4) recombinant receptors expressed in HEK293 cells.

Table 2. Association (k_{on}) and Dissociation (k_{off}) rate constants of [^{3}H]muscimol binding at room temperature in recombinant receptors expressed in HEK293 cells., mean ± SEM values.

Recombinant receptor	**k_{on} (M^{-1} x min^{-1})**	**k_{off} (min^{-1})**	**K_D (k_{off}/k_{on}) (nM)**
α6**β1**δ	2.96 ± 0.47 x 10^7	0.17 ± 0.02**	5.74
α6**β2**δ	3.42 ± 0.40 x 10^7	0.06 ± 0.01*	1.75
α6**β3**δ	6.41 ± 0.45 x $10^{7\,\#\#}$	0.13 ± 0.01	2.03

Statistical comparison of recombinant receptor k_{on} values: $^{\#\#}p < 0.01$, significantly different from α6β1δ and α6β2δ values (one-way ANOVA followed by Tukey's post hoc test). Statistical comparison of recombinant receptor k_{off} values:**p < 0.01, significantly different from α6β2δ value; *p < 0.05, significantly different from α6β3δ value (one-way ANOVA followed by Tukey's post hoc test)

3. DISCUSSION

This study aims to investigate the binding kinetics of [^{3}H]GABA and [^{3}H]muscimol in αβδ $GABA_A$ receptors, assess muscimol affinity and examine the difference in its binding properties to the recombinant α6β1δ, α6β2δ and α6β3δ $GABA_A$Rs.

3.1 GABA slows binding kinetics in recombinant αβδ $GABA_A$Rs

It is clearly shown in the present study that δ subunit in $GABA_A$R combinations have a role in slowing the rate of [^{3}H]GABA association and dissociation from recombinant receptors. On the other hand, γ2 subunit in $GABA_A$R combinations leads to faster association and dissociation. The impact of slow dissociation for δ subunit was similarly shown for [^{3}H]muscimol on native and recombinant αβδ $GABA_A$Rs (Taina, 2013). [^{3}H]GABA association rate constant (k_{on}) for α6β2δ is only 18% of the corresponding α6β2γ2 value. These findings are in correspondence with the studies on Xenopus oocytes that showed a significant low GABA efficacy on α6β2δ recombinant receptors. GABA-stimulated maximal currents for α6β2δ were only 21% of α6β2γ2 maximal currents (Hadley & Amin 2007). Chandra et al. (2010) reported that the behavioral effects of muscimol in gene-modified mouse lines were dependent on the presence of α6, α4 and δ subunits. However, the slow dissociation contribution to muscimol behavioral effects is less probable since it is similarly observed for [^{3}H]GABA on α6β2δ recombinant receptors.

3.2 Muscimol has high affinity to δ containing $GABA_A$Rs

It has been found that muscimol has high affinity binding sites in cerebellar α6βγ2 and especially in α6βδ receptors (Quirk et al., 1995, Korpi et al., 2002). The slow and prolonged nature of tonic inhibition mediated by extrasynaptic δ-containing receptors (Mody, 2001) correlates with the slow dissociation of both [^{3}H]GABA and [^{3}H]muscimol. The half-life of ligand-receptor interaction is known to be long for muscimol (Curran et al. 2002) and in the present study, [^{3}H]muscimol dissociation rate constant (k_{off}) in α6β2δ is even 7 times lower compared to [^{3}H]GABA. The equilibrium dissociation constant (K_D), which is

inversely proportional to affinity, is determined by the ratio of dissociation rate constant k_{off} and association rate constant k_{on} ($K_D = k_{off}/ k_{on}$). The association rate of [^{3}H]muscimol to δ-containing receptors is slow. This clearly concludes that muscimol's remarkable high affinity to δ-containing $GABA_A$Rs is due to its slow dissociation, which explains its moderate "superagonism" behavior.

As muscimol is a conformationally restricted $GABA_A$-analog, similarly with the endogenous GABA, it binds in zwitterionic form (Figure 6.) through two receptor-associated agonist binding sites (Chandra et al., 2010). Muscimol structure is more rigid than GABA due to its isoxazole ring. However, the polarized amino and carbonyl groups of muscimol interact with the same binding site residues as amino and carboxyl groups of GABA. This was explained by molecular docking methods suggesting that water is bound to the GABA agonist orthosteric binding pocket in addition to muscimol (Bergmann et al., 2013; Petersen et al., 2014).

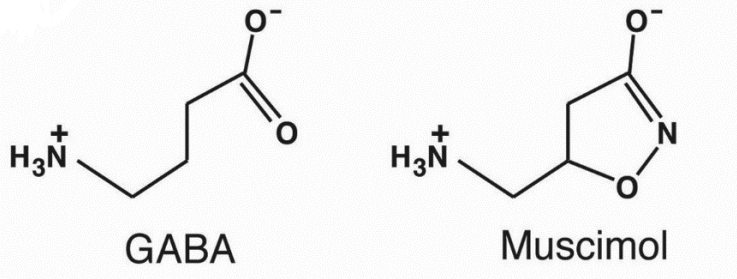

Fig. 6. GABA and muscimol structures in zwitterionic form (Krogsgaard-Larsen et al., 1979)

An earlier study reported fast association and dissociation of muscimol binding to αβγ2 recombinant receptors (Taina., 2013). However, drastic effects on [^{3}H]muscimol association and dissociation are observed when γ2 subunit is replaced by δ subunit. The stoichiometry of αβδ receptors and its subunit assembly has not been determined precisely. Nevertheless, γ2 to δ subunit substitution from 2α:2β:γ2 to 2α:2β:δ is confirmed in Barrera et al. (2008). $GABA_A$ agonist binding sites are located at the two β(+)α(-) interfaces (Sigel and Buhr, 1997). 2α:2β:δ forms two $GABA_A$ agonist sites, one at each β(+)α(-) interface. Hence, delta subunit eventually interacts with the two βα subunit pairs by forming α(+)δ(-) and δ(+)β(-) interfaces with its adjacent subunits (Figure 7). The role of δ subunit in [^{3}H]muscimol slow association and dissociation and its

high affinity is still unclear: whether muscimol acts as an antagonist, or it reactivates the receptor after deactivation without dissociating from the receptor. However, there is an evidence that agonist dissociation rates correlate inversely with binding affinity (Jones et al., 2001) supporting the claim that muscimol's slow dissociation is the reason behind its high affinity to δ-containing $GABA_A$Rs.

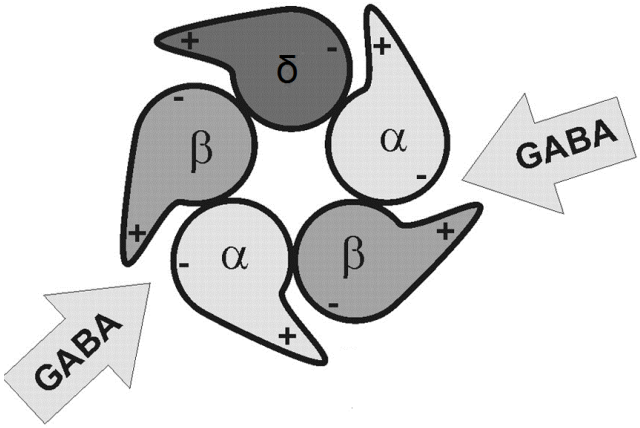

Fig. 7. $GABA_A$ agonist binding site at β(+)α(-) interfaces of αβδ $GABA_A$Rs (Modified from Ramerstorfer, J. et al., 2011)

3.3 The role of Beta subunit variant in binding properties and receptor function

3.3.1 Ligand sensitivity and efficacy

α6$β_{1-3}$δ recombinant receptors show distinct binding kinetics profiles confirming the role of $β_{1-3}$ subunits in the different binding properties of extrasynaptic δ containing $GABA_A$Rs. The remarkable slow dissociation of [^{3}H]muscimol from all three receptor subtypes confirms the studies indicating α6$β_{1-3}$δ high affinity state being independent of β subunit (Hadley & Amin, 2007) and supports the role of delta subunit in the slow binding kinetics discussed earlier. Nevertheless, this doesn't exclude the role of different β subunits in terms of ligand sensitivity to δ containing $GABA_A$ receptors. Electrophysiologically, β1 and β3 containing α6βδ receptors are more sensitive to GABA and picrotoxin binding than α6β2δ receptors. (Hadley & Amin 2007; Karim, 2012)**.** Similarly, [^{3}H]muscimol dissociation rates for β1 and β3 containing α6βδ recombinant receptors does

not significantly differ in this study and are significantly faster than for α6β2δ recombinant receptors. Hence, there may be a relation between slower dissociation from β2 containing α6βδ receptors and their lower sensitivity to $GABA_A$ ligands which needs further confirmatory electrophysiological studies.

The differences in [^{3}H]muscimol association (k_{on}) and dissociation (k_{off}) rate constants in α6β_{1-3}δ recombinant receptors was clearly due to the difference in β1, β2 and β3 subunits. Accordingly, these subunits are directly associated in the binding site itself. From a thermodynamic prospective, the relatively slower dissociation behavior of [^{3}H]muscimol in α6β2δ combinations suggests that the free energy difference between the bound and unbound states is higher in α6β2δ compared to α6β1δ and α6β3δ combinations.

The reciprocal of dissociation rate constant, known as the drug-target residence time ($\tau = 1/k_{off}$), has been shown to often predict *in vivo* efficacy better than binding affinity (Copeland et al., 2007; 2016; Pan et al., 2013). However, due to the longer drug-receptor occupancy, low dissociation rates may lower the therapeutic index (median toxic dose/median effective dose) for drug candidates. [^{3}H]Muscimol dissociation rate constant (k_{off}) is significantly lower for α6β2δ recombinant receptors given that there was no significant difference in [^{3}H]muscimol affinity to α6β2δ and α6β3δ. Hence, muscimol may show higher efficacy on α6β2δ receptors despite the fact that GABA showed low efficacy to the same receptors in previous studies (Karim, 2012). However, confirmatory studies are needed to compare muscimol-induced maximum response from native and recombinant extrasynaptic α6βδ receptors.

3.3.2 Alcohol sensitivity, anesthesia and sedation

This study highlighted the difference in binding properties between α6β1δ, α6β2δ and α6β3δ recombinant receptors which is consistent with their distinguished receptor functions discussed in the literature. The faster association of [^{3}H]muscimol to the exceptionally GABA-sensitive α6β3δ recombinant receptor (Storustovu & Ebert, 2006) compared to α6β2δ may be linked to β3 subunit role in mediating the *in vivo* effects of ethanol and general anesthetics. Microinjections of muscimol in the rat pedunculopontine tegmental

nucleus (Samson & Chappell, 2001a) and medial prefrontal cortex (Samson & Chappell 2001b) decreased ethanol self-administration, though the effect was seen at different doses.

In contrast to relatively high ethanol concentrations needed to potentiate GABA responses in αβγ subunit combinations, GABA responses in δ-containing receptors are sensitive to considerably lower ethanol concentrations (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). The high alcohol sensitivity of tonic GABA currents has been demonstrated in a number of studies showing that tonic currents in dentate granule cells, as well as in cerebellar granule cells, but not in cells that do not express the δ subunit, are enhanced by low doses of ethanol (Carta et al., 2004; Wei et al., 2004; Hanchar et al., 2005; Liang et al., 2006).

Studies showed that the inhibition of α6β3δ and α4β3δ $GABA_A$Rs was enhanced by ethanol at low concentrations of 3 mM as mild intoxication effects on humans were experienced (Wallner et al., 2003; Hanchar et al., 2004). Noting that this concentration is 3.6 times lower than the blood alcohol content (BAC) driving limit in Finland (10.9 mmol/L). These enhancements were antagonized by Ro15-4513 reversing ethanol low concentration effect on these receptors. (Wallner et al., 2006). According to Hanchar et al. (2006) some benzodiazepine derivatives, including Ro 15-4513 that have been shown to act as an ethanol antagonists at $GABA_A$ receptors (Suzdak et al., 1986), bind to α6β3δ receptors. These benzodiazepines are “silent” having no effect on GABA responses. However, Ro 15-4513 is able to block ethanol potentiation of GABA responses in α6β3δ receptors (Wallner et al., 2006). On the other hand, 10 fold decrease in ethanol sensitivity was observed for β2 subunit containing αβδ $GABA_A$Rs (Wallner et al., 2003). However, the matter remains controversial as several research groups were not able to replicate these finding for ethanol at low concentrations (≤ 30 mM) (Borghese et al., 2006). Several research groups suggested that δ subunit do not contribute in high alcohol sensitivity of recombinant receptors or tonic currents (Borghese et al., 2006; Yamashita et al., 2006; Borghese & Harris, 2007; Korpi et al., 2007). To justify, transfections with different cRNA ratios of α6:β3:δ in Xenopus oocytes produced at least four functional recombinant receptors with different stoichiometry for δ subunit

exposing variance in electrophysiological kinetic measurements (Hadley & Amin 2007; Baur et al., 2009). Some studies indicate the importance of phosphorylation state (presence of protein kinase C-delta) on ethanol stimulated GABA responses in the hippocampus (Choi et al., 2008) and in fact PKC phosphorylates α4 and β3 but not δ subunit in the hippocampus (Abramian et al., 2010).

A study by Jurd (2002) on mice showed how propofol and etomidate anesthetic effects can be lost by producing N256M point mutation in β3 subunit. This means that β3-subunit is possibly expressed more readily than β1 or β2 in α6βδ/α4βδ extrasynaptic $GABA_A$ receptors being essential targets for ethanol and general anesthetics (Wallner et al., 2003). In contrast, sedation but not anesthesia was mostly abolished in the case of β2 (N265S) mutant mice (Reynolds et al., 2003). Additionally, the sedative and hypnotic drug, methaqualone, was more effective in activating α6β2δ receptors than α6β3δ and showing negative modulation for α6β1δ receptors (Hammer, 2015). This reveals the role of β2-subunit in arbitrating the sedative effects of anesthetics.

3.3.3 Beta subunit amino acid residues and binding kinetics

There was no evidence supporting that α6β1δ would differ from α6β2δ in terms of [^{3}H]muscimol association or α6β3δ in terms dissociation. On the other hand, the association and dissociation to α6β2δ and α6β3δ are different although the M2 domain of both β2 and β3 subunits have the same amino acid residue (asparagine) at position 265 (15′) (Wingrove et al., 1994). Hence, the results suggest that residue at 265 is not involved in association and dissociation differences of $GABA_A$ receptors containing different beta subunits. However, a domain in δ subunit amino acid sequence that confers high agonist sensitivity has been identified (You & Dunn, 2007). The importance of this segment on slow kinetics of [^{3}H]muscimol needs to be studied.

3.4 Extrasynaptic δ-containing receptors as potential targets for drug development

Understanding different $GABA_A$ receptor subtypes in terms of their distinctive functional and pharmacological properties is essential for drug development. Subtype-selective drugs make it possible to modulate specific physiological functions with no undesirable side effects and less development of tolerance.

Functional characteristics of extrasynaptic αβδ receptors differ from those of αβγ2 receptors. αβδ receptors have high affinity for GABA, insensitivity to benzodiazepines and high sensitivity to neurosteroids and Zn^{2+} (Semyanov et al., 2003; Mortensen & Smart, 2006; Stórustovu & Ebert, 2006). However, they offer a potential novel target for drug development. Several positive modulators acting selectively on α4/6βδ receptors have already been discovered (Wafford et al., 2009; Lewis et al., 2010; Hoestgaard-Jensen et al., 2010). One of the two imidazo[1,2-a]pyridines, DS1, acts as an agonist and positive allosteric modulator, while the other (DS2) acts as positive modulator of α4/6βδ receptor subtypes (Wafford et al., 2009). Positive allosteric modulators of dihydropyrimidinone class are also selective for αβδ-type receptors, although the potencies of the compounds are quite low (Lewis et al., 2010). AA29504, a close analogue to the β potassium channel opener retigabine, increases the potency and efficacy of GABA on α4β3δ receptors *in vitro* (Hoestgaard-Jensen et al., 2010). In addition, AA29504-induced activation of αβδ receptors has been shown to reverse recognition memory deficits in a rat phencyclidine model of schizophrenia (Damgaard et al., 2011).

Extrasynaptic δ-containing receptors mediate tonic inhibition in the brain. Augmentation of tonic inhibition is needed in overexcited states like in epileptic seizures. Several lines of evidence suggested a role for malfunctioning δ-containing receptors in epilepsy (Lewis et al., 2010). Ganaxolone, a neurosteroid, is an example of drug under development to treat seizures as well as the developmental disorder Fragile X Syndrome (Lozano et al., 2014). It acts as a selective modulator for extrasynaptic δ-containing $GABA_A$ receptors (Brickley and Mody, 2012). Furthermore, THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol, known as gaboxadol), a partial $GABA_A$ agonist, acts on αβδ

receptors as a superagonist (Krogsgaard-Larsen et al., 2004). THIP at high concentrations increases the duration of longer channel openings and their frequency on αβδ receptors resulting in longer burst durations. Gaboxadol has been shown to improve sleep quality as a very promising novel hypnotic (Ebert et al., 2008), but due to adverse effects its development as hypnotic drug was discontinued. Nevertheless, gaboxadol is currently under study for its therapeutic potential in Angelman syndrome and Fragile X syndrome since both disorders are associated with deficiencies in $GABA_AR$ mediated tonic inhibition (Lozano et al., 2014; Egawa et al., 2012).

3.5 Methodical considerations and limitations

Long incubation times (15 min, 30 min) may induce proteolysis in recombinant receptors homogenates and affect the overall binding. EDTA was included in cell detaching buffer in order to inhibit the proteolytic enzymes at the cell harvesting step. However, EDTA was not included in the assay buffer as it can effect [^{3}H]muscimol and [^{3}H]GABA binding results. Upon the termination of [^{3}H]muscimol or [^{3}H]GABA incubation, filtration and rinsing steps took a total time of 15 s. During this, dissociation of the binding can still take place which may affect the results especially at the short time points (15 s, 30 s). In order to minimize error, the washing buffer (10 mM Tris-HCl) was kept on ice bath to keep its temperature as low as possible (Taina, 2013).

Ligand binding does not occur exclusively to the expressed $GABA_ARs$ on HEK293 cells. Other binding may occur on undefined areas in the biological samples or on the glass fiber filters. Hence, specific binding was determined indirectly by measuring the non-specific binding and total binding independently. Moreover, similar to the typical electrophysiological measurements, the binding kinetics experiments were conducted at room temperature for better correlation. Ligand-receptor binding at low temperature leads to even slower association and dissociation compared to the results obtained.

The association and dissociation curves are usually biphasic and may represent two forms of mono-liganded receptor or two populations of binding sites such as

high and low affinity binding sites (Agey and Dunn, 1989). In binding assays we used low [^{3}H]muscimol concentration (10 – 20 nM), hence most binding was observed at the high affinity state. Time points used were limited (7 for association and 6 for dissociation) making the curve fitting more convenient in "one binding site" model. That's why only one rate constant was calculated for association and dissociation reflecting the rates in one phase only.

An interesting observation was noted with the harvested HEK293 cells suspended in buffer containing glycerol 43% and stored at -20 ºC for 24 h. [^{3}H]Muscimol binding assays showed higher DPM values indicating more binding with the transfected cells pretreated with glycerol. This effect was seen in all three α6β$_{1-3}$δ recombinant receptors. A recent study by Gupta et al. (2017) showed the role of lipids in stabilizing the oligomeric state of membrane proteins. This suggests that glycerol is able to induce changes in the hetero-oligomeric GABA$_A$Rs expressed in HEK293 cells leading to higher affinity state. However, this observation was only seen with [^{3}H]muscimol hence studies are needed for other ligands (e.g. [^{3}H]EBOB, [^{3}H]Ro15-4513 and [^{35}S]TBPS) to confirm the role of glycerol in the binding affinity to different GABA$_A$ recombinant receptor binding sites.

4. SUMMARY AND CONCLUSION

This study measured [^{3}H]muscimol binding kinetics in α6β$_{1-3}$δ recombinant receptors expressed in HEK293 cells. Radiolabeled ligand binding assays showed distinct association and dissociation profiles in all three investigated recombinant receptors. The association rate of [^{3}H]muscimol was highest in α6β3δ recombinant receptors while the dissociation rate in α6β2δ was lowest. [^{3}H]Muscimol equilibrium dissociation constant was highest for α6β1δ reflecting its relative lower affinity state. This study confirms the role of β subunits in the different binding properties of extrasynaptic δ-containing GABA$_A$Rs. Further electrophysiological studies are needed to reveal how different β subunits affect binding site of αβδ GABA$_A$Rs in in terms of ligand sensitivity and efficacy. This leads to an improved pharmacological characterization of extrasynaptic αβδ receptors and possible development of selective allosteric modulators with therapeutic significance for alcoholism, anesthesia and epilepsy.

5. MATERIALS AND METHODS

5.1 Cell culture

Human Embryonic Kidney cells known as HEK293 were used for their high transfection efficiency and expression of recombinant proteins. This cell line is widely used in neuroscience studies and was initially produced by culturing normal HEK cells with sheared adenovirus 5 DNA (Graham et. Al., 1977). Cell culture medium was prepared initially by adding 13.7 g DMEM powder (Dulbecco's modified Eagle's medium, Gibco, Gaithersburg, MD) and 3.7 g sodium bicarbonate in each 1 liter of Milli-Q water. The components were solubilized with magnetic stirrer and pH was measured using Single Pore pH electrode (Hamilton, Weilheim, Germany) and adjusted with HCl to reach pH of 7.0. The medium was then sterile filtrated (Millipore Steripak™ Filters, Merck KGaA, Darmstadt, Germany) before the addition 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD; USA), 50 000 U/l penicillin and 50 mg/l streptomycin (Sigma- Aldrich, St Louis, MO, USA).

HEK293 cells with a passage number of 6 where retrieved on ice from liquid nitrogen storage tank. The frozen tube contained the cells suspended in DMEM, 10% FBS and 10% dimethyl sulfoxide (J. T. Baker, Austin, TX, USA). The sample was quickly thawed in a water bath at 37°C and put on ice with addition of 1.5 ml of FBS. The tube was then centrifuged (1600 rpm for 2 min) before discarding the supernatant with pipetting. Cell pellet was suspended in 2 ml of warm (37 °C) culture medium and transferred to 100 mm cell culture dish (Corning, New York, USA). The cells were then grown in a total volume of 10 ml culture medium in cell culture incubator at a temperature of 37°C, and 5% of carbon dioxide.

When 70-80% confluency was reached (2 - 3 days), the cells were divided to grow in a 3 fold of its growth surface area. First, the old culture medium was pipetted out. The cells were then detached from the bottom of the plate using hypertonic salt solution (40 mM Tris-HCl, 1 mM EDTA and 250 mM NaCl, pH 7.4) warmed to 37°C in a water bath. The mixture of cultured cells and hypertonic salt solution were eventually centrifuged (1600 rpm for 2 min) and

supernatant was discarded. A warmed fresh culture medium (37°C) was then added on the cell pellet to have homogenous suspension that was transferred evenly to a new 100 mm cell culture dishes and completed with fresh culture medium reaching a total volume of 10 ml. Growth conditions in the incubator where the dishes are placed remain the same with a temperature of 37°C, and 5% of carbon dioxide.

5.2 Plasmid DNA transformation and growth of bacteria

A compatible and competent DH5α *E.coli* strain was transformed with pRK5 plasmid expressing antibiotic resistance gene and containing the needed rat $GABA_A$ receptor cDNA (α6, β1, β2, β3, γ2 or δ) (Schofield et al, 1987; . Shivers et al., 1989; Ymer et al., 1989; Lüddens et al., 1990).

The plasmid ligation mixture (1 µl) was pipetted into Eppendorf tube on ice bath followed by addition of 100 µl DH5α *E.coli* thawed on ice and the mixture was kept on ice for 20 min. Heat shock was induced by incubation in 42°C water bath for 90 seconds and then transferred back to the ice bath for 5 min. The bacterial culture was then plated using a L-shaped spreader, on a 90 mm ampicillin-containing Lysogeny broth (LB) agar (Biokar diagnostics, Beauvais, France) culture plate (Sterilin, Newport, England) and grown for 12 h in a non-sterilized incubator at 37°C. An individual colony was then picked with a disposable plastic loop and added to separate tube with 3 ml of ampicillin containing LB media (Yeast extract 5 g, Tryptone 10 g, NaCl 10 g, Milli-Q water 1 liter and ampicillin 100 mg) leaving it to grow with shaking for 8 h (37°C, 250 rpm). The tube contents were then added to ampicillin-containing LB media (500 ml) and incubated in a flask with shaking for 12 h (37°C, 250 rpm). The transformed bacteria were then centrifuged (6000 rpm, 30 mins. 4°C) after which the plasmids were isolated and purified.

5.3 Plasmid purification

Plasmids were purified by NucleoBond Xtra Maxi Plasmid and Xtra Maxi EF DNA Purification kits. (Macherey-Nagel GmbH & CO.KG, Düren, Germany) as per user manual instructions. The bacterial cell pellet was resuspended in RES buffer and lysed by a sodium hydroxide/ SDS treatment with LYS buffer.

Proteins, as well as chromosomal and plasmid DNA were denatured under these conditions. RNA was degraded by DNase-free RNase A. A neutralization buffer (NEU), containing potassium acetate, was added to the lysate, causing sodium dodecyl sulphate (SDS) to precipitate as KDS (potassium dodecyl sulfate) and pulling down proteins, chromosomal DNA, and other cellular debris. The potassium acetate buffer also neutralizes the lysate. Plasmid DNA are reverted to its native supercoiled structure and remained in solution.

The lysate was then cleared and loaded onto the equilibrated acidic column filter (pH 6.5). The anion exchange column consists of hydrophilic, macroporous silica beads functionalized with positively charged methylaminoethanol (MAE) in which under acidic pH conditions it permits the negatively charged phosphate backbone of plasmid DNA to bind with high specificity. Plasmid DNA was then eluted from the column with an alkaline elution buffer (pH 9.0) to neutralize MAE charge resulting in DNA separation from the column to be collected in a tube.

The eluted plasmid was precipitated with 0.7 volumes of room temperature isopropanol and loaded onto a special silica membrane filter by means of a 30 ml syringe. The membrane was washed with 70% ethanol (4 ml) and then dried by pressing air through the filter. Elution of pure DNA was carried out by loading 400 µl of slightly alkaline low salt buffer TRIS (5 mM Tris/HCl, pH 8.5) in a 1 ml syringe and passing through the silica membrane for collection in Eppendorf tube. The eluted plasmid DNA was dissolved in 450 µl of sterile Milli-Q water and added to 1 ml of 70% ethanol and 50 µl of 3 M sodium acetate (pH 5.2) to precipitate the DNA. The tube was then centrifuged (16 000 x *g* for 10 min) and the supernatant was discarded. The pellet was left to dry for one hour and eventually dissolved in 1 ml of sterile Milli-Q water before final storage in -20°C. A sample of 2 µl was taken to determine the concentration using UV spectrophotometry at a wavelength of 260 nm.

5.4 Gel electrophoresis

The procedure is performed to validate the purified pRK5 plasmids and ensure that the analyzed plasmid contains the required cDNA encoding for the specific $GABA_A$ receptor subunit. Agarose gel concentration of 1 % was prepared by dissolving 0.5 g of agarose in a flask containing 50 ml of 1X TRIS-borate-EDTA (TBE) buffer (50 ml). The contents were dissolved under heat and kept to cool down to 40°C before careful addition of ethidium bromide (1 µl). The gel was poured carefully on the electrophoresis chamber tray and left to harden with ensuring of no bubble formation. For sample preparation, 1 µl of 10x restriction enzyme buffer was added to two sample tubes containing 1 µl and 0.1 µl of plasmid and completed with 7 µl of sterile Milli-Q water and XbaI restriction enzyme (1µl). After an incubation of 2 h at 37°C 6-fold loading buffer (2µl) was added to each sample tube before they are pipetted to the gel wells (10 µl of 1Kb DNA ladder was used as a control). The gel including the samples were transferred in the electrophoresis chamber containing 1X TBE buffer and the electrophoresis was run at a constant voltage of 70 v for one hour. UV transilluminator was eventually used to visualize the separated DNA fragments in the gel (Figure 8).

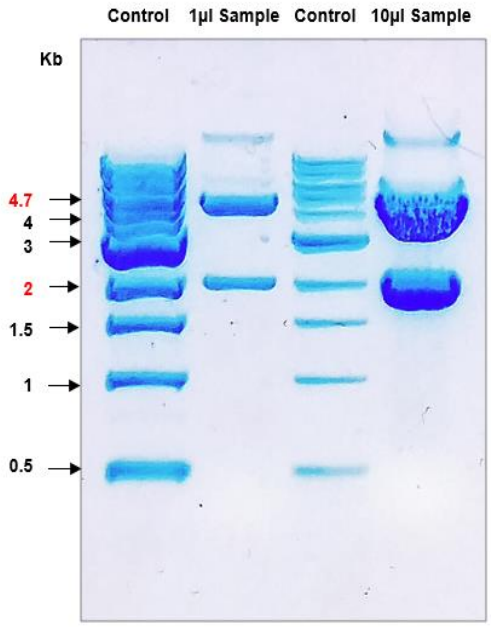

Fig. 8. Gel electrophoresis indicating the presence of rat beta1 cDNA in pRK5 plasmid digested by XbaI restriction enzyme that generate fragments sized 4.7 Kb and 2.0 Kb.

5.5 Transient expression of recombinant $GABA_AR$ subtypes

Human embryonic kidney (HEK) 293 cells were transfected with rat cDNAs (α6, β1, β2, β3, γ2S, δ) in pRK5 plasmids (Uusi-Oukari et al., 2000) under the control of CMV promoter using calcium phosphate precipitation method essentially as described by Lüddens and Korpi (1997). The procedure took place 24 h after dividing the cells in a 3 fold of its growth surface area when a confluency of 50% is reached. The plasmids coding for the different receptor subunits were used in 1:1:1 ratio for transfections containing $GABA_AR$ subtypes α6β2γ2, α6β1δ, α6β2δ and α6β3δ. For each 100 mm culture plate, a transfection mixture was prepared containing 5 µg of each subunit plasmid , 450 µl of sterile milli-Q water, 50 µl 2.5 M $CaCl_2$ solution (J. Baker, Austin, TX, USA) and 500 µl 2x HBS buffer (pH 7.00). The mixture tube was inverted 3-4 times and left to incubate for 90 seconds before pipetting dropwise around the culture plate. The transfected cells were transferred to grow in the cell culture incubator at a temperature of 37°C and carbon dioxide concentration of 5%. Fresh culture medium was replaced after 24 h and the cells were incubated for another 24 h before harvesting. The transfection efficiency of HEK293 cells was evaluated with plasmid GFP (green fluorescent protein) as its expression was visualized 48 h after transfection using a fluorescence microscope (Olympus DP70 GmbH, Hamburg, Germany). (Figure 9)

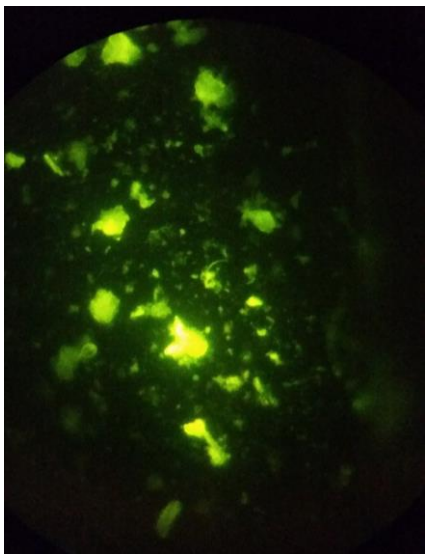

Fig. 9. The expression of GFP protein on the transfected HEK293 cells detected 48 h after transfection using a fluorescence microscope.

5.6 Cell harvesting

The cells were harvested 48 h after transfection. Culture medium was discarded and the cells were detached from the plates by pipetting in ice-cold buffer containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl and 2 mM EDTA to be collected in a centrifugation tube on ice. The cell suspension was centrifuged at 47 800 x *g* for 10 min at +4 ºC before discarding the detaching solution. The cells were either suspended in assay buffer (10 mM Tris-HCl, pH 7.4) to be used directly for binding assays or suspended in a buffer containing 10 mM Tris-HCl, pH 7.4, glycerol 43% and 0.15 M NaCl to be stored frozen at -20 ºC for 24 h.

5.7 Measurements of binding kinetics

Radiolabeled [^{3}H]GABA and [^{3}H]muscimol binding assays for HEK293 cell homogenates were performed essentially as described in Kontturi et al. (2011), Uusi-Oukari & Korpi (1989; 1990), Uusi-Oukari (1992) and in Uusi-Oukari & Maksay (2006). [^{3}H]GABA (30 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). [Methylene-^{3}H]muscimol (22 Ci/mmol) was purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). GABA was from Sigma Chemical Co. (St. Louis, MO, USA). All receptor subtypes studied were always included in the same experiment which was performed independently at least three times.

The binding of [^{3}H]GABA and [^{3}H]muscimol (10 nM/ 20 nM) were measured in 10 mM Tris-HCl, pH 7.4 assay buffer at room temperature (22 ºC) in a total volume of 300 µl. Four parallel samples were incubated with shaking for various time points (15 s, 30 s, 1 min, 2 min, 4 min, 8 min and 15 min) to measure association of the binding. These included 4 samples to determine the total binding and 4 samples to determine the nonspecific binding. In total binding tubes, 100 µl of buffer (10 mM Tris-HCl, pH 7.4) were added followed by 100 µl of cell homogenates and finally with 100 µl 3x10nM/ 3x20nM of [^{3}H]GABA or [^{3}H]muscimol. In non-specific binding tubes 100 µl of 3x100 µM GABA was added instead of buffer since GABA excess (≥1000 x) compared to muscimol displace the latter and blocks its binding to the specific binding sites.

The incubation was terminated by filtration of the samples with a Brandel Cell Harvester (model M-24, Gaithersburg, MD, USA) onto Whatman GF/B glass fiber filters (Whatman International Ltd., Maidstone, UK). The samples were rinsed twice with 4-5 ml of ice-cold assay buffer. Filtration and rinsing steps took a total time of 15 s. Air-dried filters were immersed in 3 ml of Optiphase HiSafe 3 scintillation fluid (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) and radioactivity determined in a HIDEX liquid scintillation counter (HIDEX, Turku, Finland). The DPM (disintegration per minute) values of the liquid scintillation counter indicate the amount of radioactive ligand bound to the filters.

To measure the dissociation of [^{3}H]GABA or [^{3}H]muscimol binding, the four parallel samples of cells were first preincubated at room temperature (22 ºC) in a total volume of 300 µl for 15 min with [^{3}H]GABA or [^{3}H]muscimol (10 nM/ 20 nM) in the presence of 100 µM GABA in non-specific binding tubes. The dissociation was then started by adding 100 µl of 400 µM or GABA to the incubation mixtures in the total binding tubes to reach a final 100 µM GABA concentration in all tubes. The tubes were mixed and incubations at room temperature (22 ºC) were terminated at various time points (0 s, 30 s, 1 min, 3 min, 10 min and 30 min) as described above.

5.8 Data analysis

DPM readings obtained from HIDEX liquid scintillation counter were converted to % of control values in the Excel spreadsheet program (Microsoft Corporation, Redmond, WA, USA). The mean of specific binding from all experiments were calculated for every time point. Prism 5 and Prism 6 software (GraphPad, San Diego, CA, USA) were used in statistical testing and curve fitting of the association and dissociation data. Nonlinear regression analysis were used for estimation of k_{on} and k_{off}. To calculate k_{off} ($M^{-1}min^{-1}$), the dissociation values were fitted to the one phase exponential decay equation. The association values were fitted to one phase exponential association equation to calculate k_{ob} (observed association rate constant). k_{on} (min^{-1}) was calculated with the following equation (Lundquist et al., 2005):

$$kon = \frac{kob - koff}{[radioligand(nM)]}$$

The significance difference in association and dissociation curves between recombinant receptor subtypes were assessed by two-way repeated measures ANOVA followed by Bonferroni *post hoc* test. Furthermore, the statistical comparison of k_{on} and k_{off} values were performed with two tailed unpaired *t*-test and one-way ANOVA followed by Tukey's *post ho*c test. P-values of less than 0.05 were considered significant.

6. ACKNOWLEDGMENTS

This study was supported by grants from The Finnish Foundation for Alcohol Studies. Special thank and gratitude to Mikko for his valuable support and fruitful discussions during my master's project. Thanks to: Sanna Soini for her constructive critique, Professor Markku Koulu for his mentorship and my parents for their continuous encouragement.

7. ABBREVIATIONS

CGC: Cerebellar granule cells

DGC: Dentate granule cells

DPM: Disintegration per minute

$GABA_AR$: γ-Aminobutyric acid type A receptor

HEK293: Human embryonic kidney cells 293

K_D: Equilibrium dissociation constant

k_{on}: Association rate constant

k_{off}: Dissociation rate constant

MSE: mean squared error

NAc: Nucleus Accumbens

THIP: 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol

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