universität wien

# MASTERARBEIT

Engineering of a Benzodiazepine Binding Site into $GABA_A$ Receptors Composed of Alpha1 and Beta3 Subunits

angestrebter akademischer Grad

Master of Science (MSc)


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Wien, am

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## I. Summary

$GABA_A$ receptors are the major inhibitory transmitter receptors in the brain and the site of action of many clinically important drugs, such as benzodiazepines, barbiturates, anesthetics and convulsants. The majority of $GABA_A$ receptors are composed of two alpha (α), two beta (β) and one gamma (γ) subunit. The two GABA binding sites are located at the two extracellular β-plus/α-minus interfaces whereas the binding site for benzodiazepines is located at the homologous α-plus/γ-minus interface. Despite the extensive clinical use of benzodiazepines, the molecular basis of their interaction with the $GABA_A$ receptor, as well as the protein structure of the benzodiazepine binding site is not known.

To obtain information on the structural requirements of the benzodiazepine binding site, we investigated whether it is possible to engineer a benzodiazepine binding site into α1β3 receptors, which presumably are composed of two α1 and three β3 subunits and have no high affinity benzodiazepine binding site. To achieve this task, the β3-minus side had to be converted at least partially to a γ2-minus side. Using a structural model of the diazepam-bound benzodiazepine binding site as a template, that was recently developed by our group, as well as previously published mutagenesis data, we introduced several amino acid residues of the γ2-minus side that are important for benzodiazepine binding, into the β3-minus side of an α1β3 $GABA_A$ receptor.

Benzodiazepine affinity was studied by radioligand binding assays with appropriately transfected human embryonic kidney cells, whilst modulation of the mutated recombinant receptors was investigated by the two electrode voltage clamp method in Xenopus laevis oocytes. Results indicate that by using four mutations (β3DGE) we were able to introduce a high affinity benzodiazepine binding site into the α1β3 model system. Furthermore, we were able to show that the GABA currents of the mutated recombinant receptors α1β3DG (three mutations), and α1β3DGE, could be modulated by Ro15-1788 and flurazepam.

# II. Zusammenfassung

$GABA_A$ Rezeptoren sind die wichtigsten inhibitorischen Neurotransmitter Rezeptoren des Säugetier Gehirns, und werden durch GABA (γ-Aminobuttersäure) aktiviert. Zahlreiche klinisch relevante Wirkstoffe wie Benzodiazepine, Barbiturate, Anästhetika können neben GABA an den $GABA_A$ Rezeptor binden und seine Wirkung modulieren. Obwohl diese Wirkstoffe in der Medizin eine breite Anwendung finden, ist die genaue Struktur der Bindungsstelle nicht bekannt. Dies macht ihn zu einem hoch interessanten Untersuchungsgegenstand der Neurobiologie.

$GABA_A$ Rezeptoren bestehen aus fünf Untereinheiten. Die Mehrheit der Rezeptoren sind aus einer gamma (γ), zwei alpha (α), und zwei beta (β) Untereinheiten zusammengesetzt, wobei die hoch affine Bindung von Benzodiazepinen das Vorhandensein einer gamma Untereinheit voraussetzt. Jede Untereinheit hat per Definition eine plus und eine minus Seite. Die Benzodiazepin Bindungstelle befindet sich im extrazellulären Bereich zwischen der α-plus und der γ-minus Seite dieser Untereinheiten.

Das Ziel dieser Arbeit war es, eine hoch affine Benzodiazepin Bindungsstelle in einen α1β3 Rezeptor einzubauen, der im Gegensatz zu einem α1β3γ2 Rezeptor keine solche besitzt. Der α1β3 Rezeptor besteht aus zwei α und drei β Untereinheiten. Der Reihe nach wurden wichtige Aminosäuren der γ-minus Seite (eines α1β3γ2 Rezeptors) in die β-minus Seite des α1β3 Rezeptors eingebaut. Diese Aminosäuren wurden mittels eines in unserem Labor entwickelten strukturellen Modells der Diazepam-gebundenen Benzodiazepin Bindungsstelle und mittels bereits publizierter Artikel ausgewählt.

Die Affinität von Benzodiazepinen wurde durch Radioliganden-Bindungsstudien an Membranen von HEK Zellen bestimmt die mit den entsprechenden Rezeptoren transfiziert worden waren. Die erhaltenen Resultate lassen darauf schließen, dass vier Mutationen (β3DGE) ausreichten, um eine hochaffine Benzodiazepin Bindungsstelle in die α1β3 Rezeptoren einzubauen. Die pharmakologische Charakterisierung wurde mittels einer zwei Elektroden Spannungsklemme in Xenopus laevis Oozyten vorgenommen. Die Ergebnisse zeigen, dass sich die durch Flurazepam und Ro15-1788 ausgelöste Modulation der GABA Ströme der rekombinanten Rezeptorenα1β3DG (dreifach Mutante) und α1β3DGE von der des Wildtyp α1β3 Rezeptors unterscheidet.

*Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und Ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt.*

## III. Acknowledgments

I would like to thank Prof. Werner Sieghart for his overall supervision, help and scientific advice with my master thesis. I have profited thoroughly from his excellent scientific understanding. It was a great opportunity to work in his lab.

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All my colleagues from the Sieghart Lab for their scientific input, and the pleasant time we shared.

# IV. Abbrevations

| | |
|---|---|
| **APS** | Ammoniumpersulfate |
| **Bmax** | Maximal binding site density |
| **BCA** | Bicinchoninic acid |
| **Bp** | Base pair |
| **BZ** | Benzodiazepine |
| **cDNA** | Complementary Desoxyribonucleic acid |
| **E.coli** | *Escherichia Coli* |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **GABA** | γ-Aminobutyricacid |
| **HEPES** | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| **$K_D$** | Ligand concentration where 50% of all receptors are occupied |
| **kDa** | Kilo Dalton |
| **$K_i$** | Equilibrium dissociation constant of the unlabeled ligand |
| **Min.** | Minute |
| **mM** | millimolar |
| **MOPS** | N-Morpholino propanesulfonic acid |
| **µM** | micromolar |
| **nAChR** | Nicotinic acetylcholine receptor |
| **nM** | nanomolar |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PBS** | Phosphate buffered saline |
| **pLGIC** | Pentameric Ligand Gated Ion channels |
| **PVDF** | Polyvinylidene fluoride |
| **SDS** | Sodium dodecyl sulphate |
| **Sec.** | Second |
| **SEM** | Standard error of mean |
| **TEMED** | N,N,N´,N´-tetra-methyl-ethylene-diamine |
| **TM** | Trans membrane |
| **Tris** | Tris (hydroxymethyl) aminoethane |

# 1. INTRODUCTION

## 1.1.Cys-Loop Receptor Family

$GABA_A$ receptors belong to the family of the Cys-loop pentameric ligand gated ion channel receptors (pLGIC). All members of this superfamily share a homologous 13 amino acid residue long "loop" at the extracellular domain which is flanked by a disulfide bridge (Karlin 2002; Sixma 2007). PLGIC can be divided into cation-selective excitatory receptors, such as the nicotinic acetylcholine receptors (nAChR), the ionotrophic 5-HT3 receptors and a $Zn^{2+}$ activated ion channel, and anion-selective inhibitory ones, such as the glycine receptors and the $GABA_A$ receptors (Olsen and Sieghart 2008). Members of this superfamily use similar sequences and functional domains to establish receptor structure and functionality (Olsen and Sieghart 2008): As shown in Fig.1.1, each subunit consists of an N-terminal extracellular domain region containing a cys-loop, four transmembrane α-helices, of which TM2 forms the lining of the ion channel, a large intracellular loop between TM3-TM4 and an extracellular C-terminal end.

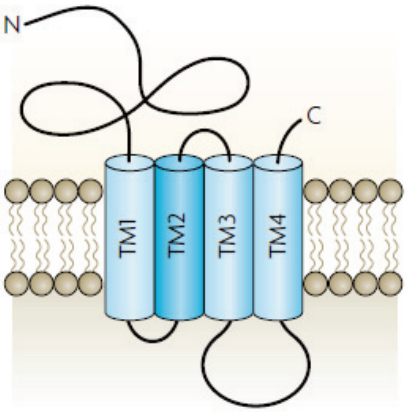


**Fig. 1.1: Membrane organization of a subunit from the pLGIC family (Jacob, Moss et al. 2008).**

Detailed knowledge about their protein structure is of great research interest, as they are pharmacologically relevant. 1988 the first structural information on near crystalline arrays of the nAChR in the electric ray *Torpedo* became available (Unwin, Toyoshima et al. 1988; Toyoshima and Unwin 1990). 2001 the crystal structure of the acetylcholine binding protein (AChBP), a water-soluble protein

homologous to the extracellular part of nAChR from snail, was published (Brejc, van Dijk et al. 2001). Then, low-resolution electron microscopy images at 4Å of the nAChR were published (Miyazawa, Fujiyoshi et al. 2003; Unwin 2005), and in 2007 the high-resolution crystal structure of the extracellular ligand binding domain of a single AChR subunit was revealed (Dellisanti, Yao et al. 2007). Recently, the first prokaryotic receptor belonging to the family of pLGIC could be crystallized and revealed an extracellular organization similar to that of the AChBP in eukaryotes (Hilf and Dutzler 2008; Hilf and Dutzler 2009). In spite of great efforts, so far it was not possible to crystallize the $GABA_A$ receptor or other receptors of the mammalian pLGIC.

All models of the extracellular and transmembrane domains of the $GABA_A$ receptor used in our laboratory, were based on the published AChBP and nAChR crystal structures and validated by computational methods (Ernst, Brauchart et al. 2003; Ernst, Bruckner et al. 2005; Richter submitted). This is possible, as the sequence identity of the subunit members of the pLGIC is 20-30% (Olsen and Sieghart 2008), although there is a significant variability in the local interface and pocket structure between the different members of the superfamily (Richter submitted). Functional homology at the secondary and tertiary level was suggested to be higher, as receptors use similar domains for channel structure, agonist binding and allosteric ligands (Olsen and Sieghart 2008). Furthermore, there is evidence that GABA and acetylcholine bind at a similar subunit interface, involving homologous amino acid residues (Olsen, Chang et al. 2004). These findings are of particular interest when revealing the ligand binding sites, possible domain interactions and conformational changes (Olsen, Chang et al. 2004) of the $GABA_A$ receptor, because of their medical implications in pharmacological drug developmental studies.

### 1.2.$GABA_A$ Receptor

Gamma-Aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the central nervous system and exerts most of its actions via $GABA_A$ receptors (Sieghart 1995). The GABAergic synaptic transmission is central to neuronal activity and information processing in the mammalian brain (Minelli,

Brecha et al. 1995). GABA is synthesized by the decarboxylation of glutamate by the enzyme glutamic acid decarboxylase (Bear 2001) as shown in Fig. 1.2, whereas glutamate is a citric acid cycle intermediate, produced in the mitochondrion. GABA is stored in vesicles of the presynaptic terminals of neurons, released into the synaptic cleft upon depolarization of the presynaptic membrane and subsequent intracellular $Ca^{2+}$ increase and binds to receptors on the postsynaptic membrane. GABA transporters, localized at neurons and glia cells, re-accumulate released transmitters into presynaptic terminals and glia cells. GABA is either re-used after accumulation into vesicles by vesicular transporters or metabolized by GABA-transaminase, for further usage in the citric acid cycle (Minelli, Brecha et al. 1995; Bear 2001).

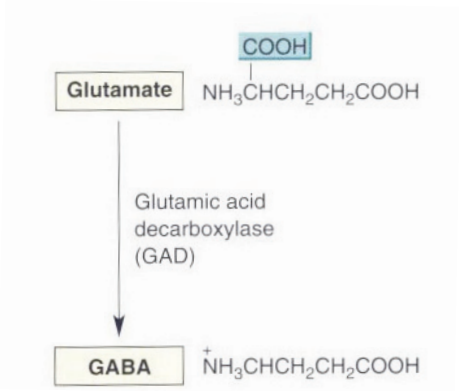


**Fig. 1.2: GABA synthesis by the decarboxylation of glutamate (Bear 2001).**

There are two GABA receptor types by which the action of GABA can be mediated: The ionotropic $GABA_A$ receptors and the metabotropic $GABA_B$ receptors. Occasionally in the literature $GABA_C$ receptors are mentioned; but these are ionotrophic $GABA_A$ receptors composed of rho (ρ) subunits (Olsen and Sieghart 2009).

$GABA_A$ receptors can be stimulated by GABA, muscimol and isoguvacine and inhibited noncompetitively by picrotoxin and competitively by bicuculline (Sieghart 1995). Ionotrophic $GABA_A$ receptors are pentameric receptors that can be composed of up to five different subunits (Tretter, Ehya et al. 1997; Klausberger, Sarto et al. 2001) that directly form an anion-selective channel. Upon binding of GABA, the receptors increase their neuronal membrane conductance for $Cl^-$ ions, and as the $Cl^-$

ion concentration within the cell is normally lower than outside, the opening of the channel forces $Cl^-$ ions into the cell (Sieghart 1995). This leads to rapid inhibitory currents causing hyperpolarization and a reduction of neuronal excitability (Olsen and Sieghart 2008). In early development as well as under conditions of disease, when the $Cl^-$ equilibrium potential is less negative than the resting membrane potential of the cell, receptor activation can increase neuronal excitability (Olsen and Sieghart 2008).

Metabotrophic $GABA_B$ receptors are heterodimeric G-protein-coupled receptors composed of $GABA_{B1}$ and $GABA_{B2}$ subunits. They can be located pre- and postsynaptically and are coupled to $Ca^{2+}$ and $K^+$ channels, respectively. Activation of presynaptic $GABA_B$ receptors can close $Ca^{2+}$ channels, leading to inhibition of transmitter release. Activation of postsynaptic $GABA_B$ receptors can activate $K^+$ channels leading to neuronal hyperpolarization (Bowery 2006). These receptors can be stimulated by GABA or (-)baclofen and inhibited by phaclofen (Sieghart 1995).

The metabotropic $GABA_B$ receptors can be distinguished from faster opening ionotrophic $GABA_A$ receptors by their different kinetics in the inhibitory post synaptic potentials (IPSPs).

### 1.3.Receptor Structure

There are in sum 19 different mammalian $GABA_A$ receptor subunit genes identified: 6α, 3β, 3γ, 1δ, 1ε, 1π, 1θ, 3ρ and furthermore there exist some subunit splice variants predominantly within the γ2 subunit: γ2L (large), γ2S (small). There is a large number of possible subunit combinations, but only very few have been unequivocally identified (Olsen and Sieghart 2008). The most abundant subunit composition of the $GABA_A$ receptor consists of one γ, two α, and two β subunits, forming the functional heteropentameric receptor, indicated in Fig. 1.3 (A) (Sieghart 1995; Tretter, Ehya et al. 1997; Klausberger, Ehya et al. 2001). The formation of pentameric receptors consisting of only one or two subunits is also possible, although with a lower assembly efficiency (Sieghart 1995; Tretter, Ehya et al. 1997).

Similar to nAChR, $GABA_A$ receptors are allosteric receptors, carrying multiple agonist-binding sites (Karlin 2002). GABA and benzodiazepines bind at subunit interfaces of the predominantly β-stranded extracellular domains, which are formed by a plus side of one, and a minus side of another subunit (Monod, Wyman et al. 1965; Ernst, Brauchart et al. 2003) as shown in Fig 1.3 (B).

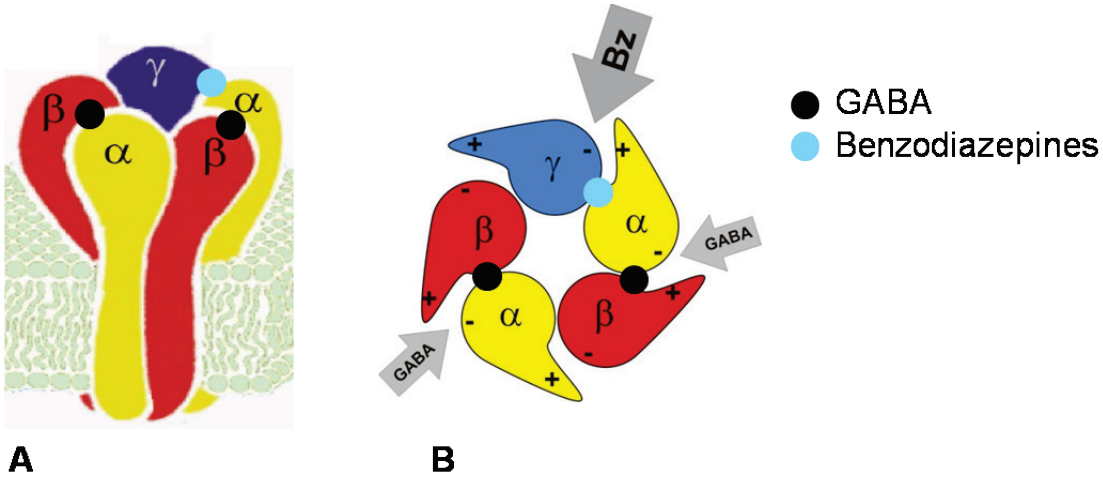


**Fig. 1.3: (A) Subunit composition of the functional heteropentameric $GABA_A$ receptor, modified from Olsen (Olsen and Sieghart 2008). (B) Model of the extracellular part of the $GABA_A$ Receptor viewed from above, with possible ligand binding sites indicated as arrows. Modified from Klausberger and Ernst (Klausberger, Sarto et al. 2001; Ernst, Bruckner et al. 2005). GABA binding sites are indicated as black, the benzodiazepine binding site as light blue circles.**

The two GABA binding sites are located at the β-plus/α-minus interface. High affinity binding of classical benzodiazepines requires the minus site of the γ2 subunit (Pritchett, Sontheimer et al. 1989) together with the plus site of one of the α1,2,3, or 5 subunits (Olsen, Chang et al. 2004). A low affinity binding site for the benzodiazepine flurazepam was additionally found at the α-plus/β-minus interface (Baur, Tan et al. 2008). Many key elements of agonist and drug action seem to be located at the interface of the minus and the plus side of two subunits (Ernst, Bruckner et al. 2005).

## 1.4.$GABA_A$ Receptor Pharmacology

$GABA_A$ receptors are the target of a large variety of clinically and pharmacologically important drugs such as benzodiazepines, barbiturates, anesthetics and convulsants. They bind at allosteric binding sites of the receptor, modulate the GABA induced current and exert their effects by initiating an allosteric transition in the protein that indirectly modifies the conformation of the agonist binding site (Changeux and Edelstein 1998; Teissere and Czajkowski 2001). There can be compounds enhancing the GABA induced currents, such as benzodiazepines which exhibit anxioloytic, anticonvulsant, muscle relaxant, sedative and hypnotic effects, or compounds reducing the action of the receptor which exhibit anxiogenic and convulsant effects (Sieghart 1995). The large pharmacological variety of compounds can be ascribed to differing binding modes at the binding pocket. Furthermore, the expression of different $GABA_A$ receptor subunits in various brain regions, results in functionally different receptor subtypes (Sieghart 1995). Depending on their subunit composition the receptors show different pharmacological and electrophysiological properties (Sarto-Jackson and Sieghart 2008).

## 1.5.Outline of the Project

The following master thesis is integrated into the Neurocypres project of the European Union. It was the objective of my master thesis to investigate whether it is possible to engineer a benzodiazepine binding site by a consecutive introduction of point mutations into hetero-oligomeric α1β3 $GABA_A$ receptors (Fig. 1.4 C). For this, an α1β3 receptor (Fig. 1.4 B) was chosen as a model system, as it has, compared to an α1β3γ2 receptor (Fig. 1.4 A), no high affinity benzodiazepine binding site but still two GABA binding sites. These are important for the pharmacological characterization with benzodiazepines, as benzodiazepines cannot open the chloride channel but only modulate GABA-induced currents.

This diploma thesis was the first step for demonstrating the feasibility of engineering a benzodiazepine binding site into a related receptor that does not contain such a site. Incorporation of such a site in receptors that can be crystallized, could then be used

as a starting point for first crystallization trials of this binding site and drug development studies.

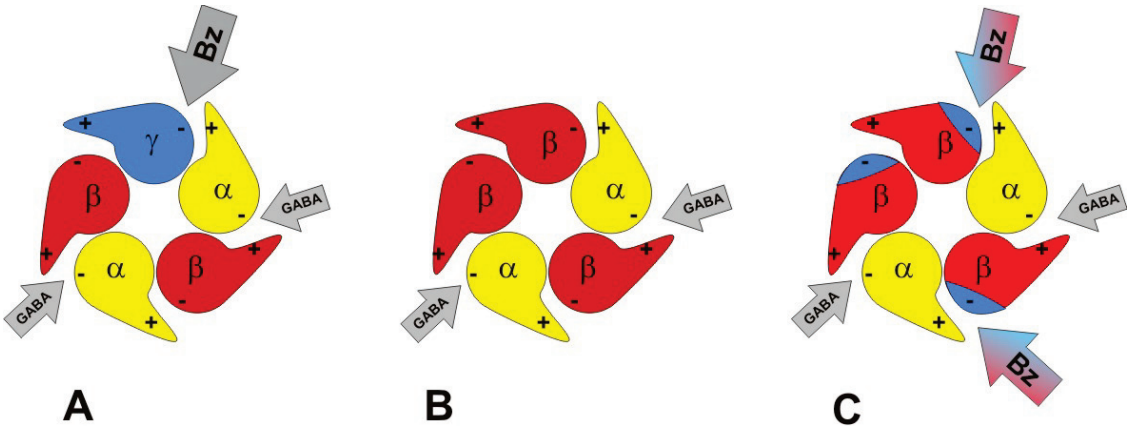


**Figure 1.4**: **Schematic structure of the extracellular domain of $GABA_A$ receptor types (top down view). (A) The wild type α1β3γ2 receptor has a benzodiazepine binding site situated at the α-plus/γ-minus interface, (B) in the wild type α1β3 receptor a β replaces the γ subunit. This receptor exhibits no high affinity benzodiazepine binding site, (C) the engineered α1β3 receptor with the introduced benzodiazepine binding sites, with important amino acids of the γ-minus side introduced into the β-minus side. Arrows indicate the location of the GABA and the benzodiazepine binding site(s).**

To achieve the goal of this thesis we engineered important amino acid residues of the γ2-minus side of the α1β3γ2 receptor, into the β3-minus side of an α1β3 receptor as Fig. 1.4 (C) shows. The amino acid residues were selected by using a structural model of the diazepam-bound benzodiazepine binding site as a template that was recently developed (Richter submitted) and previously published mutagenesis data. A total of six different mutant β3pCI constructs were generated, co-expressed in different heterologous cell systems together with wild type α1 subunits, and investigated by biochemical, electrophysiological and radioligand binding techniques.

# 2. MATERIAL AND METHODS

## 2.1. Generation of Mutated cDNA Constructs

For the cloning of mutated β3 subunits, pCI expression vectors (Promega, Madison, Wisconsin) were used. Seven mutant constructs were designed by the method of "gene SOEing" PCR technique (Horton 1993) using a β3pCI vector as template, which had a the full length of a rat β3 subunit (1421bp) incorporated in a pCI vector (Ehya, Sarto et al. 2003). The mutant products replaced parts of the wild type sequence on the vector. The *Escherichia Coli* strain XL1-Blue (Stratagene, La Jolla, California) was used for plasmid amplification and isolation. Transformations were performed as published previously (Fuchs, Zezula et al. 1995). The wild type β3pCI vector, as well as the resulting mutant products, were digested with the restriction enzymes PstI and XhoI (Promega, Madison, Wisconsin) for subsequent ligation.

### 2.1.1. Gene SOEing, PCR

The Polymerase Chain Reaction (PCR) method of "gene SOEing" (Gene Splicing by Overlap Extension) was used to generate single mutations or chimeric constructs by the overlap extension of two complementary sequences using the PCR technique. With the technique of "gene SOEing", synthetic oligonucleotide primers containing the recombinant sequence become incorporated into the final PCR product (Pogulis, Vallejo et al. 1996).

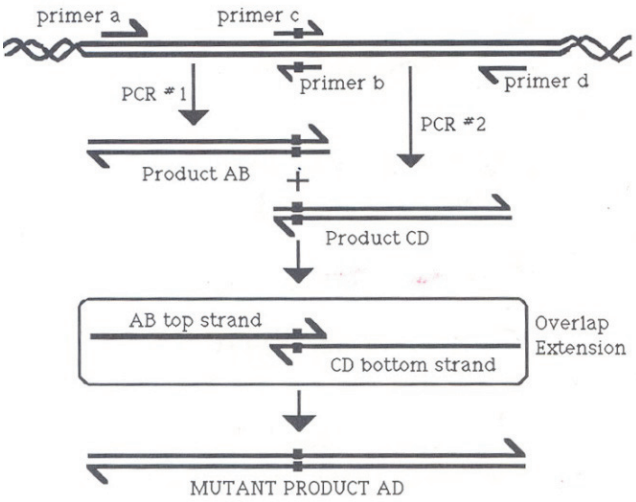


**Figure 2.1: The method of Gene Splicing by Overlap Extension (Horton 1993)**

This method was described by Horton (Horton 1993). Recombinant primers, complementary to the open reading frame (ORF) of the β3 subunit within the template DNA were designed for nucleotide synthesis in 5' to 3' direction and contained a 3'priming region which binds to the β3pCI template DNA (with an annealing temperature of 60°C), and a 5'overlapping region (for the overlap of the two internal primers) containing the mutation/chimeric sequence. In addition, two flanking primers were chosen near the restriction sites PstI or XhoI. In two separate PCR reactions, flanking primers (a, d) and recombinant primers (b, c) generated two overlapping fragments AB, CD (Fig. 2.1), which were fused in a second round of PCR (Pogulis, Vallejo et al. 1996). In the second round of PCR the 3'upper strand of the AB fragment could hybridize with the 5'end of the lower strand of the fragment CD (with an annealing temperature at 56°C). High fidelity DNA polymerase (Hoffmann-La Roche, Basel, Swiss) proceeded to complete the mutant product, as the overlapping strands were themselves primers, resulting in a full-length mutant product AD (Horton 1993; Pogulis, Vallejo et al. 1996).

### 2.1.2. Digestion and Purification of DNA

After the generation of the mutant constructs, PCR products were purified using the GFX purification kit of DNA from agarose gel (GE Healthcare, Chalfont

St. Giles, Buckinghamshire). The eluates were digested with the restriction enzymes PstI and XhoI according to the manufacturer's protocol (Promega, Madison, Wisconsin). Linear DNA fragments were purified from the solution containing the restriction enzymes, using the GFX purification kit for DNA. In parallel, wild type β3pCI vectors were digested with the respective restriction enzymes and purified from a preparative agarose gel using the GFX purification kit for DNA.

### 2.1.3. Preparative Agarose Gel for Band Separation

Preparative agarose gel for band separation by length was used to purify mutant PCR products and digested wild type β3pCI vectors. Agarose (Invitrogen, Carlsbad, California) gels (0.01g/ml) were prepared in 1xTBE (10xTBE: 890mM Tris, 890mM boric acid, 31mM EDTA) with the addition of ethidium bromide (stock: 10 mg/ml) to a final concentration of 0.5 µg/ml. Electrophoretic separation of DNA fragments was performed at 100V.

### 2.1.4. GFX Purification of DNA from Gel

DNA was purified with a GFX Purification Kit. Following electrophoresis of the DNA, the respective agarose band was excised from the gel and dissolved by the addition of capture buffer at 60°C. A GFX MicroSpin column was incubated with the solution and centrifuged. The column was washed with 500µl Tris-EDTA buffer (10mM Tris-HCl with pH 8.0, 1mM EDTA, 80% Ethanol) and centrifuged again. DNA was eluted with TE buffer (10 mM Tris-HCl with pH 8.0, 1mM EDTA). For a detailed protocol see website of GE Healthcare.

### 2.1.5. GFX Purification of DNA from Solution

To 100µl of the respective solution 500µl capture buffer was added. After transfer to the GFX MicroSpin column, sample was processed as described above.

### 2.1.6. Ligation of DNA Fragments

Ligation of the linear DNA fragments and the linearized vectors was performed using the protocol of the Rapid DNA Ligation Kit (Hoffmann-La Roche, Basel, Swiss). Following the ligation of the mutated DNA fragments and the linearized wild type vector, XL1-Blue *E.coli* competent cells were transformed with the ligation mix.

### 2.1.7. Chemically Competent XL1-Blue E.coli Cells

- **Generation**

30ml SOB medium (2% Bacto-Trypton, 0.5% Bacto-Yeast Extract, 10mM NaCl, 2.5mM KCl) was prepared with a tetracycline concentration of 12.5 µg/ml (stock 5 mg/ml). Frozen glycerol cultures of *E.coli* XL1-Blue bacterial cells were dissolved in the medium with an inoculating loop, and incubated for 16 hours at 37 °C. Subsequently, a suspension culture was prepared with 60ml SOB adding 0.6ml $Mg^{2+}$stock (10mM $MgCl_2$, 10mM $MgSO_4$) together with 0.5ml of the overnight culture, and grown at 37°C until reaching an optical density of 0.45 ($OD_{600}$). The culture was centrifuged at 1000x*g*, 15 min, 4°C, then resuspended in 12.5ml FSB (10mM $KCH_3COO$, 100mM KCl, 45mM $MnCl_2$, 10mM $CaCl_2$, 3mM Cobalt hexamine trichloride, 10% Glycerol, with pH of 7.0, sterile filtered), incubated on ice for 15 min. and centrifuged again at 1000x*g*, 15 min, 4°C. The pellet was resuspended in 3.8ml FSB together with 140µl DMSO, following a second addition of 140µl DMSO after 5min. Finally the cell suspension was aliquoted and stored at -70°C.

- **Transformation**

Aliquots of 200µl of frozen competent XL1-Blue bacterial cells were thawed on ice for 10min. About 1ng of the ligated plasmid DNA was added to the competent cells and incubated for 30min. After a heat shock of 90sec. at 42°C, and an incubation on ice for 2min., 800µl of SOC medium (SOB, plus 10µl/ml 2M Glucose) were added and the mixture was incubated for 1hour at 37°C. Finally the transformed cells were plated on a LB/Amp-agar plates (1% Bacto-Trypton, 0.5% Bacto-Yeast

Extract, 85mM NaCl, 1.5% Agar, adding 100 µg/ml ampicilin). As a control, the wild type β3pCI plasmid was transformed and plated in parallel to the mutant plasmids.

### 2.1.8. Screening for Recombinant Clones

In order to confirm successful ligation and transformation of the mutant or chimeric plasmids, a PCR screen was performed. For this, several colonies were picked from the LB/Amp agar plate and resuspended in 100µl TE buffer. From this, 40µl of cell suspension were transferred into 1ml LB/Amp solution (1% Bacto-Trypton, 0.5% Bacto-Yeast Extract, 85mM NaCl, adding 100 µg/ml ampicillin) and grown at 37°C. The remaining cell suspension in the TE buffer was subjected to a heat shock for 5min at 95°C, was incubated on ice and centrifuged at 1600x*g*, 2min. The supernatant was used for the PCR screen with the standard protocol of using Taq DNA polymerase (Qiagen, Valencia, California). After PCR, two positive clones were selected and grown in LB/Amp solution for 16hours.

### 2.1.9. Purification of DNA with the Mini-Kit from QIAgen

The DNA was prepared using the QIAgen Plasmid Mini-Kit according to the manufacturer´s protocol (Qiagen, Valencia, California). DNA concentrations were calculated by measuring the photometric absorption at 260nm. DNA samples containing the appropriate mutations were verified by sequencing.

### 2.1.10. Generation of Glycerolstocks

Storage of transformed bacterial cultures was prepared by the addition of a glycerol freezing solution (gylcerol) to the fresh grown cultures in a 1:1 ratio, and frozen at -70°C.

### 2.1.11. Purification of DNA with the Maxi-Kit from QIAgen

Following the confirmed introduction of mutations into the β3pCI vectors, positive clones were grown for 16hours in LB/Amp solution and then purified according to the protocol of Qiagen Plasmid Maxi-Kit (Qiagen, Valencia, California).

## 2.2. Transfection of HEK 293 Cells

Transformed Human Embryonic Kidney cells (HEK 293) were cultured as described before (Tretter, Ehya et al. 1997). HEK cells were grown in DMEM medium (Gibco, Carlsbad, California) with 1x MEM non essential amino acids (Gibco, Carlsbad, California), 10% fetal calf serum (BioWhittaker, Walkersville, Maryland), 2mM L-Glutamine, 100U/ml penicillin G sodium and 100µg/ml streptomycin sulfate added additionally. Cells were grown in the incubator at 37°C/5% $CO_2$ until reaching a cell number of 2 x $10^6$ per plate. With a total of about 20µg cDNA/per plate, HEK cells were co-transfected with wild type α1 together with wild type β3, γ2 or mutated β3 subunits in a ratio of 1:1:1, as described previously (Sarto-Jackson, Furtmueller et al. 2007). The transfection was performed using the calcium phosphate precipitation method (Chen and Okayama 1988). A glycerol shock was performed to increase the rate of transfection. Cells were harvested after 48 hours of growth, in order to allow receptor expression.

## 2.3. Antibodies

The antibodies α1(1-9), or β3(1-13) or β3(345-408) were generated in rabbits recognizing the epitopes according to amino acids sequences 1-9 of the mature α1 subunit or 1-13 or 345-408 of the mature β3 subunit, respectively. The antibodies were affinity purified as described previously (Tretter, Ehya et al. 1997; Jechlinger, Pelz et al. 1998). All antibodies were tested for their specificity and lack of cross-reactivity (previous experiments, not shown).

## 2.4. Immunoprecipitation of Receptors Expressed at the Cell Surface

The medium of transfected HEK cells was removed from the culture dishes. Cells were washed once in 1x Phosphate Buffered Saline (PBS: 4.3mM $Na_2HPO_4$, 2.7mM KCl, 140mM NaCl, 1.5mM $KH_2PO_4$, with a pH 7.3) and incubated for 5min. with 5mM EDTA/PBS to detach cells from dishes. DMEM was added and the cell suspension was centrifuged at 1000x*g* for 5min at 4°C. The pellet was resuspended in DMEM and the cell suspension of three plates was incubated with either 35µg of an α1(1-9) or β3(1-13) antibody, both directed against the N-terminal region of the respective subunits. Incubation was performed for 45 min. at 37°C to specifically label $GABA_A$ receptors expressed at the cell surface. Following incubation, cells were repeatedly centrifuged and washed in PBS to remove unbound antibody. With an IP-Low solution (50mM Tris-HCl, 150mM NaCl, 1mM EDTA pH 8.0, 0.5% Triton X-100) containing the detergent TritonX-100, proteins were extracted by shaking for 1 hour. Following a centrifugation step at 150.000x*g* at 4°C for 20min, the supernatant containing the proteins was collected. Immunoprecipitin (Invitrogen, Carlsbad, California) was prepared as described previously (Tretter, Ehya et al. 1997), diluted together with 5% milk powder in IP-low buffer and added to the supernatant shaking for 2 hours at 4°C, in order to precipitate the bound antibodies. The precipitate was centrifuged at 10.000x*g* for 5min at 4°C and the supernatant of the precipitated proteins was dissolved in NuPAGE sample buffer (Invitrogen, Carlsbad, California) for subsequent SDS-PAGE and Western blot analysis. It was previously shown that under these conditions the redistribution of antibodies could be excluded (Klausberger, Fuchs et al. 2000).

## 2.5. SDS-PAGE and Western Blot Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a method where proteins are denaturated by the detergent SDS, and separated according to their molecular mass in a polyacrylamid matrix (Neville and Glossmann 1974). A 10% separating gel (25% separating gel buffer containing 1.5M Bis-Tris at a pH 6.4, 30% acrylamid whereof is 0.8% N, N'-methylen-bis-acrylamid, 10% SDS, 1% Tetraethylmethylendiamin (TEMED), 10% APS) was prepared in a NOVEX gel

cassette (Invitrogen, Carlsbad, California). A 5% stacking gel (25% stacking gel buffer containing 0.5M Bis-Tris at a pH 6.4, 30% acrylamid whereof is 0.8% N, N'-methylen-bis-acrylamid, 10% SDS, 1% Tetraethylmethylendiamin (TEMED), 10% APS) was casted over the separating gel, concentrating the proteins into a thin starting zone for subsequent electrophoresis. After polymerization, the gel cassette was placed into the running chamber and samples of previous immunoprecipitation experiments were loaded onto the gel together with 1µg/µl of prestained MagicMark XP Western Standard and an unstained SeaBlue2 marker (both Invitrogen, Carlsbad, California). The outer chamber was filled with 1x MOPS (20x stock: 1M MOPS, 1M Tris, 20mM EDTA, 2% SDS), while the inner chamber was filled with 1x MOPS adding 0.25% NuPAGE antioxidant (Invitrogen, Carlsbad, California). The gel ran for 45min. at 200V, 125mA. After the electrophoresis was completed, the gel cassette was cracked open, and washed in 1x transfer buffer (10x stock: 479mM Tris, 386mM Glycine, 0.5% SDS). In parallel, a PVDF (polyvinylidene fluoride) Immobilon-P membrane (Milipore, Billerica, Massachusetts) was washed first in methanol, then dd$H_2O$ and finally in 1x transfer buffer. The blotting sandwich was built as follows: filter paper (Whatman, Atlanta, Georgia) pre-wetted in 1x transfer buffer – PVDF Immobilon-P membrane – the SDS-PAGE gel – pre-wetted filter paper, and was blotted for 60min at 20V, 58mA (per Gel). Afterwards the membrane was washed with blocking buffer (1.5% milk powder, 0.1% Tween-20) and incubated with digoxigenin labeled α1(1-9) or β3(345-408) antibodies, shaking for 16 hours at 4°C. Membranes were then washed repeatedly with blocking buffer, and incubated at room temperature with secondary anti-digoxigenin, alkaline phosphatase coupled antibodies for subsequent chemiluminescence detection (Roche, Mannheim, Germany). Following incubation, the membrane was washed repeatedly in blocking buffer and subsequently in assay buffer (1mM MgCl2, 25mM dietholamine at a pH 10.0). For visualization, the substrate of the alkaline phosphatase CDP-Star (Applied Biosystems, Bedford, Massachusetts) was added and analyzed by the Fluor-S Multimager (X-OMAT UV, Kodak). As shown previously, the bands of the α1 subunits appeared at 50kDa, while β3 subunits between 50-55kDa (Tretter, Ehya et al. 1997).

## 2.6. Chemicals

Compounds were purchased from the following sources: [N-Methyl-$^3$H]Ro15-1788 (87.0 Ci/mmol) (Perkin-Elmer Life Sciences, Wellesley, Massachusetts), diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), flurazepam (7-Chloro-1-(diethylamino)ethyl-5-(2-fluorophenyl)-3H-1,4-benzodiazepin-2(1H)-one dihydrochloride) (Sigma Aldrich, St. Louis, Missouri), Ro15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate) (Tocris Bioscience, Bristol, UK).

## 2.7. Two Electrode Voltage Clamp

Mature female Xenopus laevis (Nasco, Fort Atkinson, Wisconsin) were anaesthetized (0.17 % Tricaine, Sigma Aldrich, St. Louis, Missouri), decapitated and their ovary was removed. Xenopus laevis oocytes were prepared as described previously (Sigel, Baur et al. 1990). Stage 5 to 6 oocytes with the follicle cell layer were singled out of the ovary using a platinum wire loop. Cells were then injected with 3ng cDNA solution endocing for wild type or recombinant α1and β3 subunits (ratio 1:1), or α1, β3, and γ2 subunits (ratio 1:1:5), and incubated at +18°C in Bath Solution (10mM Hepes, pH 7.4, 88mM NaCl, 1mM KCl, 2.4mM $NaHCO_3$, 0.82mM $MgSO_4$, 0.34 $Ca(NO_3)_2$, 0.41mM $CaCl_2$ and 100 units/ml penicillin, 100µg/ml streptomycin). After at least 36 hours (depending on the subunit composition), cells were defolliculated with collagenase IA (Sigma Aldrich, St. Louis, Missouri) and trypsin inhibitor Type I-S from soybean (Sigma Aldrich, St. Louis, Missouri) and incubated in Xenopus ringer solution (5mM Hepes, pH 7.4, 90mM NaCl, 1mM $MgCl_2$, 1mM KCl, 1mM $CaCl_2$) for another 24 hours. Two electrode voltage clamp measurements were performed at a holding potential of -60mV using electrodes with a resistance between 1-4MΩ . Xenopus Ringer solution was used as perfusion buffer, and 2M KCl as inner electrode solution. Compounds were measured at a GABA concentration eliciting 3-5% maximal GABA current. The compounds flurazepam and Ro15-1788 were investigated in detail. Both are benzodiazepines which are known to bind with high affinity at the α-plus/γ-minus interface of the $GABA_A$ receptor. Between the applications of the different compounds, the perfusion system was washed with DMSO. The percentage of modulation of the GABA currents was

compared to GABA control currents. All data analysis was performed using GraphPad Prism 4 program (GraphPad Software Inc., San Diego, California).

### 2.8. Receptor Binding Studies

The experimental procedure was adapted from previously described protocols (Ogris, Poltl et al. 2004; Sarto-Jackson, Furtmueller et al. 2007). Frozen membranes of transfected HEK cells were thawed and solubilized in TC50 buffer (50mM Tris, 15.5mM Citric acid at pH 7.4). Membranes were homogenized by Ultra-Turrax and centrifuged at 150000x*g* at 4°C for 20 minutes. After resuspension in TC50 they were repeatedly ultra-turraxed and kept on ice. Protein concentrations were measured with a BCA Protein Assay kit (Thermo Fisher Scientific Inc., Rockford, Illinois) and compared to a bovine serum albumin (BSA) standard curve generated in parallel ranging from 100-1000µg/µl. For the binding assay, membranes were incubated for 90 min. at 4°C in a 1ml solution, containing TC50 buffer, 150mM NaCl and different concentrations of [$^3$H]Ro15-1788 (87Ci/mmol, DuPont, Boston, Massachusetts) ranging from 0.1-20nM, in the presence or absence of 100µM diazepam or Ro15-1788. Whatman GF/B filters (Whatman, Maidstone, Kent) were pre-wetted with ice cold water, the incubated membrane samples were filtered through and then rinsed with 3.7ml of ice cold TC50. Filters were then dissolved in the liquid scintillation cocktail Ultima Gold (Perkin Elmer, Wellesley, Massachusetts) and placed into the liquid scintillation counter over night. All data analysis was performed using GraphPad Prism 4 program (GraphPad Software Inc., San Diego, California). The respective maximal number of binding sites of the specific ligand per mg/protein ($B_{max}$) and the dissociation constant ($K_D$) describing the affinity of wild type and recombinant receptors were determined. The experiment was repeated once (α1β3γ2 wild type receptors) or thrice (α1β3DGE recombinant receptors) using cell membranes from different transfections.

For the inhibition studies, membranes were incubated with [$^3$H]Ro15-1788 in the range of the $K_D$ of the respective receptors, which were determined in previous receptor binding studies. Membranes of wild type α1β3γ2 were incubated with 10nM, and α1β3DGE recombinant receptors with 30nM of tritium labeled [$^3$H]Ro15-

1788 together with TC50 buffer, 150mM NaCl, different concentrations of unlabeled flurazepam (3nM– 30µM), in a total volume of 1ml for 90min. at 4°C. Nonspecific binding was determined in the presence of 100µM Ro15-1788 and subtracted from total [$^3$H]Ro15-1788 binding, in order to obtain specific binding. Samples were filtered and analyzed as described above.

# 3. RESULTS

## 3.1. Generation of Mutated β3 Subunits

Amino acids of the minus side of the γ2 subunit, which have previously been demonstrated to be important for benzodiazepine binding (Sigel and Buhr 1997; Wingrove, Thompson et al. 1997; Kucken, Wagner et al. 2000; Ernst, Brauchart et al. 2003; Kucken, Teissere et al. 2003; Ernst, Bruckner et al. 2005; Richter submitted) were introduced into the minus side of a β3 subunit (Fig. 3.1), using a pCI plasmid containing the full length of a rat β3 subunit as a template. The selection of amino acids was based on predictions from modelling studies (Ernst, Brauchart et al. 2003; Ernst, Bruckner et al. 2005; Richter submitted) and on published mutagenesis data. All mutations introduced, together with their positions on the "loops" are summarized in Fig 3.1 (B). Of these so-called "loops" only three are loops in the structural sense (Elliott 2003), because the six segments that form the binding pocket were identified prior to their 3D structure. The other three turned out to be pieces of beta strand (Ernst, Brauchart et al. 2003; Ernst, Bruckner et al. 2005). For the full length sequence alignment of the β3 together with the γ2 subunit see the appendix.

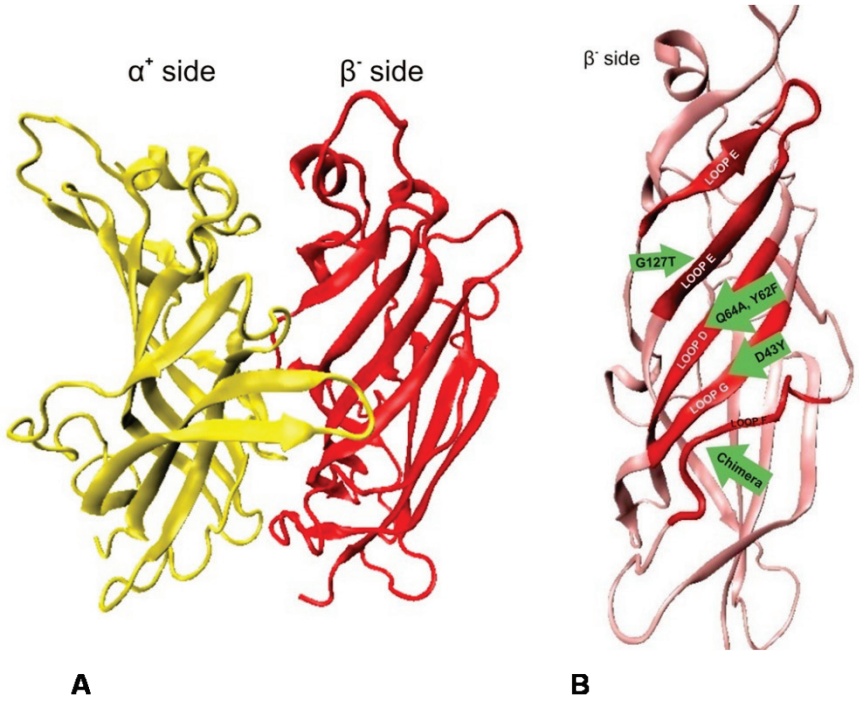


**Fig. 3.1: (A) Model structure of $GABA_A$ receptor extracellular domains of an α1β3 receptor. The α-plus/β-minus interface is viewed from the side. (B) Positions of the**

**introduced mutations on the respective "loops" (D, G, E, F) of the β-minus pocket lining interface indicated with green arrows (modified after Ernst, 2009, unpublished).**

In „loop" D two amino acid residues of the γ2 minus side were incorporated into the β3 minus side. The first amino acid which was chosen for our studies was the residue γ2F77, identified as a key determinant of the benzodiazepine binding (Buhr, Baur et al. 1997; Wingrove, Thompson et al. 1997; Sigel, Schaerer et al. 1998) replacing the homologous β3Y62. The second residue mutated in our studies was the amino acid γ2A79 (Kucken, Wagner et al. 2000) replacing the homologous β3Q64. It was shown that longer amino acid side chains would cause a decrease in binding affinities of benzodiazepines (Kucken, Teissere et al. 2003). Both residues are located adjacent to each other on a β-strand and are presumably pocket lining residues. Subsequently, the amino acid γ2Y58 (Buhr, Baur et al. 1997; Kucken, Wagner et al. 2000) of „loop" G was introduced in the β3 subunit, replacing β3D43 at the homologous position. This residue was chosen due the observation, that all γ subunits of the $GABA_A$ receptor have a tyrosine (Y) and all β subunits have an aspartic acid (D) at the homologous amino acid residue position. In „loop" E the amino acid γ2T142, neighbouring the γ2F77 residue in the anti-parallel sheet, was shown to be important for high affinity benzodiazepine binding (Pritchett, Sontheimer et al. 1989; Mihic, Whiting et al. 1994; Wingrove, Thompson et al. 1997), and therefore replaced the β3G127. In 2007, Sancar et al. published an amino acid stretch in „loop" F of the γ2 subunit, required for binding of the imidazopyridine zolpidem, which is structurally different from classical benzodiazepines (Sancar, Ericksen et al. 2007; Hanson, Morlock et al. 2008). As the residues of „loop" F are not well defined, two different chimeras were designed for our studies. For this, we substituted either a longer sequence (amino acids γ2(186-197)) or a shorter sequence (amino acids γ2(186-192)) for the homologous amino acid residues of the β3 subunit.


**Figure 3.2: Table of mutated β3pCI constructs in „loops" G, D, E and F. Mutations were subsequently introduced: construct β3D1pCI carries the mutation Y62F, β3D2pCI the mutations Y62F and Q64A, β3DGpCI the mutations Y62F, Q64A and D43Y, β3DGEpCI the mutations Y62F, Q64A, D43Y and G127T, β3DGE-F7pCI the mutations Y62F, Q64A, D43Y, G127T and a shorter sequence (amino acids γ2(186-192)), and β3DGE-F12pCI the mutations Y62F, Q64A, D43Y, G127T and a longer sequence (amino acids γ2(186-197)) of the β3 subunit with the homologous amino acid residues of the γ2 subunit.**

Thus, a total of six different mutated β3pCI constructs were generated (Fig.3.2). The first construct β3D1pCI carries the mutation Y62F. Here, the amino acid residue Tyrosine (Y) at the position 62 (of the mature peptide) in the β3 subunit was changed to the homologous amino acid residue Phenylalanine (F) of the γ2 subunit. Construct β3D2pCI carries the mutations Y62F and Q64A, β3DGpCI the mutations Y62F, Q64A and D43Y, β3DGEpCI the mutations Y62F, Q64A, D43Y and G127T,

β3DGE-F7pCI the mutations Y62F, Q64A, D43Y, G127T and a chimeric sequence containing the seven amino acids γ2(186-192), and β3DGE-F12pCI the mutations Y62F, Q64A, D43Y, G127T and a chimeric sequence containing the twelve amino acids γ2(186-197).

Wild-type or mutated β3 subunits were then co-expressed together with a wild type α1 subunit, to form a functional pentameric α1β3 $GABA_A$ receptor (Baumann, Baur et al. 2001).

## 3.2. Surface Precipitation Indicates a Comparable Expression and Assembly of Wild Type and Mutated α1β3 $GABA_A$ Receptors

To investigate the level of expression and assembly of mutated β3 subunit constructs, receptors on the cell surface expressed in HEK cells were investigated by the method of cell surface precipitation. HEK cells were co-transfected with wild-type α1 together with wild-type or mutated β3 receptors. Receptors at the cell surface were labeled with β3(1-13) (Fig. 3.3 A) or α1(1-9) antibodies (Fig. 3.3 B). Labeled receptors were extracted and precipitated by the addition of Immunoprecipitin. SDS-PAGE and Western blot analysis was performed using digoxigenin labeled β3(345-408) or α1(1-9) antibodies.

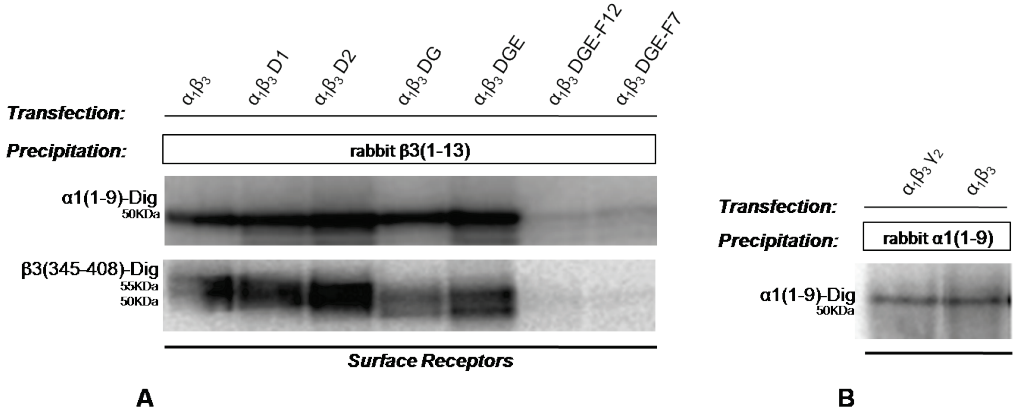


**Fig. 3.3: (A) Cell surface expression of $GABA_A$ receptors containing wild-type α1 and β3 or mutated β3 subunits. HEK cells were co-transfected with α1 and β3, β3D1, β3D2, β3DG, β3DGE, β3DGE-F12 or β3DGE-F7 subunits. $GABA_A$ receptors expressed at the**

**surface were immunolabeled by an incubation of intact cells with β3(1-13) antibody. Receptors were then extracted, precipitated by Immunoprecipitin, and subjected to SDS-PAGE and Western blot analysis using digoxigenin-labeled α1(1-9) or β3(345-408) antibodies. The complete experiment was performed once. (B) Cell surface expression of $GABA_A$ receptors containing wild type α1, β3 or α1, β3 and γ2 subunits. $GABA_A$ receptors expressed at the surface were immunolabeled by α1(1-9) antibody and Western blot analysis was performed using digoxigenin-labeled α1(1-9) antibody.**

Recombinant α1β3D1, α1β3D2, α1β3DGE receptors showed a comparable expression to the wild type α1β3 receptors when precipitated with β3(1-13) antibodies and detected with α1(1-9) digoxigenin labeled antibody (protein band at 50kDa) and with β3(345-408) digoxigenin labeled antibody (protein band at 50-55kDa) (Fig.3.3 A). There is evidence, that in addition to α1β3 receptors, β3 homopentameric receptors are present in transfections (Tretter, Ehya et al. 1997). Recombinant receptors composed of α1 and β3DG subunits, seemed to exhibit a lower degree of β3 homopentameric receptors, because there is a comparable expression of α1 subunits, but a reduced expression of β3 subunits. The increased staining of α1β3D2 receptors when detected with β3(345-408) digoxigenin labelled antibody could indicate the additional expression of β3 homopentamers. To confirm these assumptions, the experiments would have to be repeated. Recombinant receptors α1β3DGE-F12 and α1β3DGE-F7 showed a dramatically decreased expression on the cell surface, suggesting that subunit assembly or receptor formation in α1β3DGE-F12 and α1β3DGE-F7 receptors is interrupted to some extent, as similar results were obtained when precipitation of total receptors was performed (results not shown). As shown in Fig.3.3 B and previous experiments, there is no significant difference in expression at the cell surface between wild type α1β3γ2 and α1β3 receptors (Sarto-Jackson, unpublished data).

### 3.3. Electrophysiological Investigation of Recombinant Receptors $\alpha_1\beta_3$DG and $\alpha_1\beta_3$DGE with Flurazepam and Ro15-1788 (Flumazenil)

Functional investigations were performed with wild type α1β3, α1β3γ2 and recombinant $\alpha_1\beta_3$DG, $\alpha_1\beta_3$DGE receptors using two electrode voltage clamp

electrophysiology. For that, Xenopus laevis oocytes were co-injected with cDNA of wild type α1 and β3DG or β3DGE subunits into the cell nucleus. Two days after injection GABA elicited currents were recorded at a holding potential of -60mV. First GABA-dose-response curves were measured. Then compound dose-response curves were measured at a GABA concentration eliciting 2-5% of the maximal GABA current. Recombinant receptors α1β3DG and α1β3DGE were characterized in detail with the compounds flurazepam (Fig. 3.5) and Ro15-1788 (Fig. 3.6). Their chemical structure is shown in Fig.3.4 below.

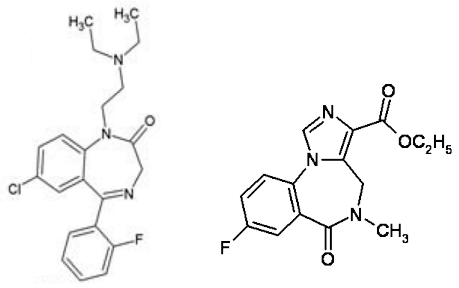


**Fig.3.4: Chemical structure of investigated benzodiazepines. The core chemical structure is a benzene ring fused with a diazepine ring. (A) Flurazepam, containing a diethylamino nitrogen side chain on N1. (B) Ro15-1788, has an imidazo-ring together with an ester group as side chain.**

The concentration-effect curve of flurazepam for the respective receptors is shown in Fig. 3.5 (A-C). In the compound dose-response curve (Fig. 3.5 D) the percentage of the modulation of GABA currents is plotted against increasing concentrations of the respective compound. Compounds were dissolved in DMSO to maximum solubility and were then adequately diluted to result in a maximum of 0,1% of DMSO in the final superfusion solution.

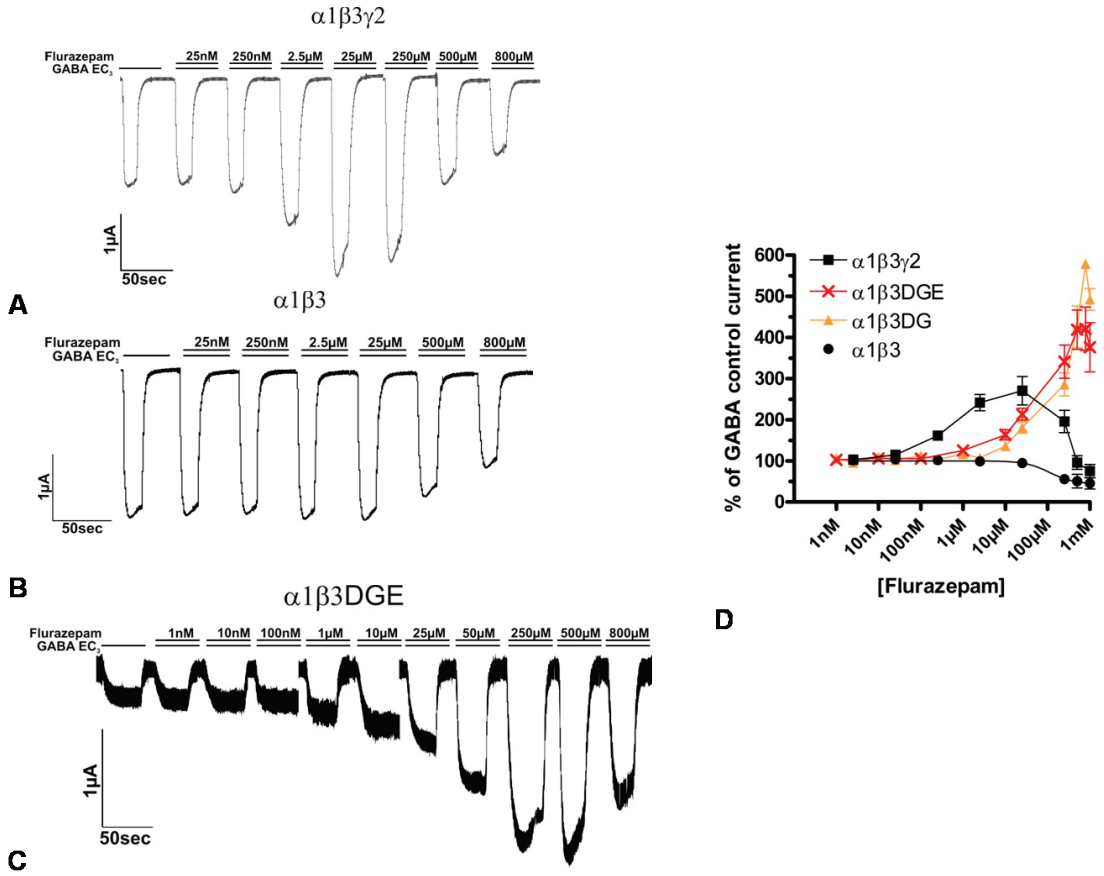


**Figure 3.5: Concentration-effect curve of flurazepam for (A) α1β3γ2, (B) α1β3 and (C) α1β3DGE receptors. (D) Xenopus laevis oocytes were injected with cDNA and investigated by the two electrode voltage clamp method. Modulation of GABA currents in recombinant α1β3γ2 (■, n=10), α1β3 (●, n=8), α1β3DG ( ▲, n=7) and α1β3DGE (×, n=8) receptors by flurazepam.**

GABA-elicited currents of flurazepam (1nM to 800µM) were recorded. As shown before (Walters, Hadley et al. 2000), flurazepam exerts two effects on α1β3γ2 wild type receptors (Fig 3.5 A). The first effect, the positive modulation of the GABA currents up to the concentration of 25µM is exerted via the benzodiazepine binding site, which is located at the α-plus/γ-minus interface. In this concentration range flurazepam has no modulatory effect on α1β3 receptors (Fig. 3.5 B). At higher concentrations (starting from 25µM) a second negative modulatory effect occurs at both wild type α1β3 and α1β3γ2 receptors (Fig. 3.5 A, B), which was shown to be exerted through a binding site located at the α-plus/β-minus interface (Baur, Tan et al. 2008). When applied to the recombinant mutated receptors α1β3DG (not shown) and α1β3DGE (Fig. 3.5 C), flurazepam positively modulates GABA currents, which

is in contrast to α1β3 receptors. The positive modulation of GABA currents in both recombinant receptors is significant compared to α1β3 receptors starting from 10µM flurazepam ($p<0.005$), while the difference in modulation between α1β3DG and α1β3DGE receptors is not significant ($p>0.05$). These results demonstrate that the α1β3 character of recombinant receptors was changed towards an α1β3γ2 receptor by the introduction of the respective mutations. At higher concentrations ranging from 25µM up to 500µM, both mutated receptors exert a strong positive modulation, whereas a decrease in positive modulation is induced only at concentrations at 800µM. Compared to α1β3γ2 wild-type receptors, not only the compound dose-response curve, but also the negative modulatory effect on GABA current potentiation at high concentrations, seems to be reduced and shifted to the right (Fig. 3.5 D). In Fig. 3.5 (C) a complete concentration-effect curve of α1β3DGE demonstrates this effect.

The effects of Ro15-1788 (1nM to 100µM) on GABA-elicited currents were recorded in wild type α1β3γ2 (Fig 3.6 A), α1β3 (Fig 3.6 B) receptors, or recombinant α1β3DG (not shown), and α1β3DGE (Fig 3.6 C) receptors.

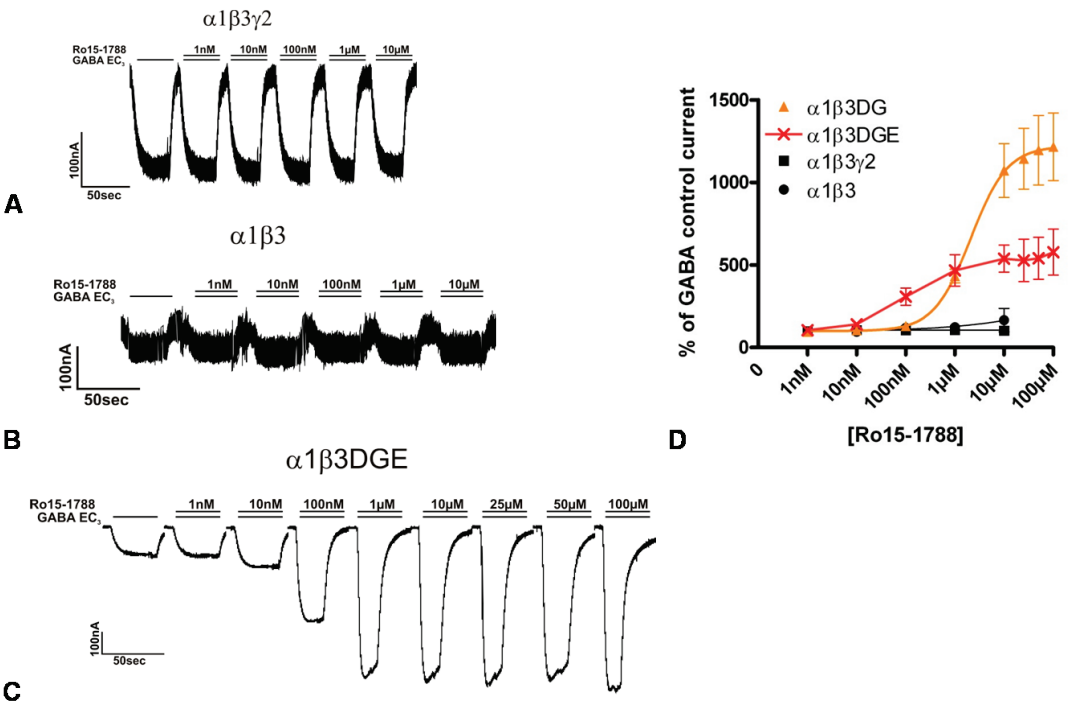


**Figure 3.6: Concentration-effect curve of Ro15-1788 for (A) α1β3γ2, (B) α1β3 and (C) α1β3DGE receptors. Xenopus laevis oocytes were injected with the respective cDNAs**

**and investigated by the two electrode voltage clamp method. (D) Modulation of GABA currents in recombinant α1β3γ2 (■, n=4), α1β3 (●, n=2), α1β3DG (▲, n=4) and α1β3DGE (×, n=6) receptors by Ro15-1788.**

It has been shown before (Hunkeler, Mohler et al. 1981; Walters, Hadley et al. 2000) that Ro15-1788 is a selective antagonist for the high-affinity benzodiazepine binding site at $GABA_A$ receptors and therefore shows no significant positive modulatory effect on wild-type receptors (Fig 3.6 A, B). Interestingly the two recombinant receptors α1β3DG and α1β3DGE exert allosteric effects different from those of the wild type receptors. At both recombinant receptors Ro15-1788 positively modulates GABA currents, as the exemplary concentration-effect curve for α1β3DGE demonstrates (Fig. 3.6 C). The recombinant receptors α1β3DGE show already a significant onset potentiation of GABA currents at 10nM ($p<0.005$) Ro15-1788, compared to α1β3DG at 1μM ($p<0.005$) Ro15-1788 (Fig. 3.6 D). The dose-response curve of Ro15-1788 at α1β3DGE receptors reaches a plateau of saturation between 1μM-10μM of Ro15-1788 (3.6 D), and slightly enhances the GABA current between 10μM-100μM. The positive modulation of GABA currents by Ro15-1788 in α1β3DG recombinant receptors is much stronger than in α1β3DGE receptors.

## 3.4. Binding of [$^3$H]Ro15-1788 to HEK Cell Membranes Containing Recombinant $\alpha_1\beta_3$DGE Receptors

Because of the highly promising electrophysiological results obtained with recombinant α1β3DGE receptors, suggesting the introduction of a high affinity binding site (significant potentiation of GABA current at 10nM ($p<0.005$), half maximal potentiation at about 100nM for Ro15-1788), we were interested in determining the affinity of the benzodiazepine-like binding site, which was introduced by four mutations at the minus interface of the β3 subunit. For this, membranes of HEK cells, co-transfected with wild-type α1, β3, and γ2 (Fig. 3.7 A) or α1 and β3DGE (Fig. 3.7 B) subunits were incubated with different concentrations of [$^3$H]Ro15-1788 ranging from 0.1nM to 20nM in the absence or presence of 100μM diazepam or Ro15-1788. The affinity of the radioactive ligand [$^3$H]Ro15-

1788 for wild type and recombinant receptors ($K_D$), and their respective binding site density ($B_{max}$) was determined by Scatchard-analysis (graphs not shown).

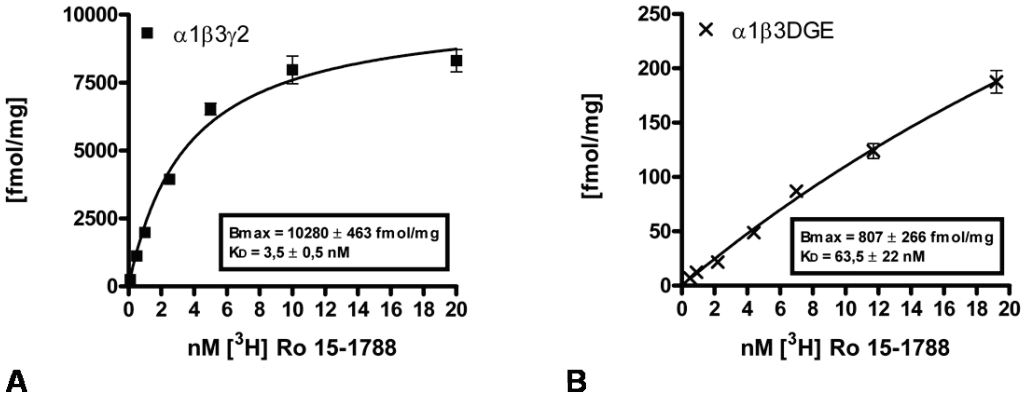


**Figure 3.7: Saturation assay of [$^3$H]Ro15-1788 binding in HEK cell membranes of (A) wild type α1β3γ2 (■) and (B) rec ombinant α1β3DGE (×) receptors. Membranes were incubated with increasing concentrations of [$^3$H]Ro15-1788 (0,1-20nM) in the absence or presence of 100µM diazepam or Ro15-1788. Membranes were filtered through Whatman GF/B filters and specifically bound radioactivity was measured. Data are presented as mean ± S.D. of one experiment performed in triplicates. Experiments were performed once (A) or three times (B) with comparable results. $B_{max}$ gives the maximal number of binding sites of the specific ligand per mg/protein. $K_D$ gives the concentration at which 50% of the receptor is occupied by the ligand.**

Graph Pad Prism analysis of saturation assays revealed, that recombinant α1β3DGE receptors exhibit a $K_D$ of 64±22 nM (Fig. 3.7 B), compared to wild type α1β3γ2 receptors which exhibit a $K_D$ of 3.7±0.5 nM (Fig. 3.7 A). $K_D$ values of recombinant α1β3DGE receptors, determined in two other experiments were 14±5 nM and 42±21 nM (mean±SD, results not shown). This indicates the presence of a high affinity benzodiazepine binding pocket, although recombinant receptors exert an affinity twenty times lower than wild type α1β3γ2 receptors. The binding site densities differed significantly, as α1β3γ2 receptors had a $B_{max}$ of 10.280±462 fmoles/mg (Fig. 3.7 A) and α1β3DGE receptors a $B_{max}$ of 807±266 fmoles/mg (Fig. 3.7 B), which suggests a mutated receptor formation of 7.5% in transfected HEK cells. In additional experiments recombinant α1β3DGE receptors exhibited $B_{max}$ values of 232±50 fmoles/mg and 159±21 fmoles/mg (results not shown). The variability of

$B_{max}$ values in these experiments probably was due to different transfection efficiencies in different experiments.

### 3.5. Inhibition of [$^3$H]Ro15-1788 Binding by Flurazepam in HEK Cell Membranes of Recombinant $\alpha_1\beta_3$DGE Receptors

To further characterize the benzodiazepine binding site of recombinant α1β3DGE receptors, competition assays were performed. The potency of flurazepam for the inhibition of [$^3$H]Ro15-1788 binding was determined. For that, membranes of HEK cells were co-transfected with wild-type α1, β3, and γ2 or α1 and β3DGE subunits and were incubated with 10nM or 30nM [$^3$H]Ro15-1788 in the absence or presence of flurazepam concentrations, ranging from 3nM to 30µM (Fig. 3.8). The equilibrium dissociation constant ($K_i$) of the unlabeled ligand was calculated by the *Cheng-Prusoff* equation: $K_i = IC_{50} / 1 + L/K_d$ (IC50… concentration of the unlabeled ligand, inhibiting the binding of the radioactive ligand by 50%; L…concentration of radioactive ligand; $K_d$… affinity of radioactive ligand to the receptor).

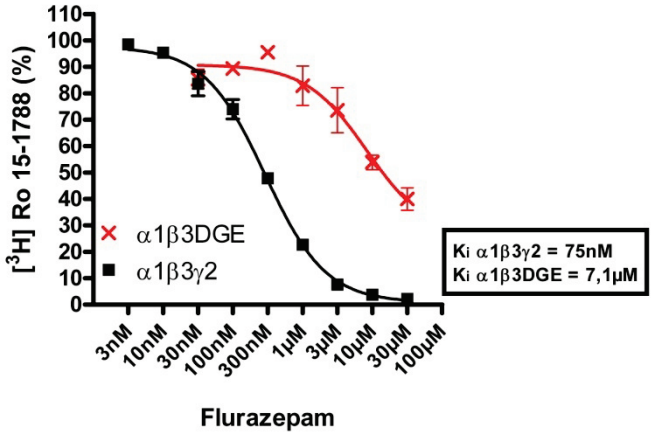


**Figure 3.8: Potency of flurazepam for inhibition of [$^3$H]Ro15-1788 binding in HEK cell membranes of recombinant α1β3γ2 (■) and α1β3DGE ( ×) receptors. Membranes were incubated with 10nM or 30nM [$^3$H]Ro15-1788 in the presence of 3nM to 30µM flurazepam. Membranes were filtered through Whatman GF/B filters and specifically bound radioactivity was measured. Data are presented as means ± S.D. of one experiment performed in triplicates and were fitted by GraphPad Prism using the equation for one site binding.**

Recombinant receptors α1β3DGE exhibited a $K_i$ of 7.1μM, compared to α1β3γ2 wild type receptors which exhibited a $K_i$ of 75nM (Fig. 3.8). This hundredfold shift suggests a reduced potency of flurazepam for the inhibition of [$^3$H]Ro15-1788.

# 4. DISCUSSION

Despite the extensive clinical use of benzodiazepines, the molecular basis of their interaction with the $GABA_A$ receptor, as well as the protein structure of the benzodiazepine binding site is not known. Recently, modelling and docking studies of our group provided the first information on a possible structure of the diazepam bound benzodiazepine binding site (Richter submitted). This structural model is supported by all experimental data available and allows structural predictions of amino acid residues important for benzodiazepine binding. In the present work, it was used for predicting which amino acid residues present at the minus side of the γ2 subunit have to be incorporated into the minus side of the β3 subunit of α1β3 $GABA_A$ receptors, to engineer a high affinity benzodiazepine binding site, that is usually formed at the α1-plus/γ2-minus interface.

## 4.1. The Diazepam-Bound Model of the $GABA_A$ Receptor Predicts Amino Acid Residues Important for Benzodiazepine Binding

Flurazepam is a positive modulator at the benzodiazepine binding site formed by the α1-plus/γ2-minus interface, and interacts with lower potency at the α1-plus/β3-minus interface causing no or a slightly negative modulatory activity (Baur, Tan et al. 2008). Together, these effects cause its biphasic modulatory action in α1β3γ2 receptors (Fig. 3.5 A). In contrast, Ro15-1788 does not bind to the α1-plus/β3-minus interface. When flurazepam and Ro15-1788 were superposed with the α1-plus/β3-minus interface it was shown that the compounds were able to exhibit an interaction similar to that at the α1-plus/γ2-minus interface with regard to the α1-plus side. However, several amino acid residues on the β3-minus side interfered with binding of Ro15-1788 and flurazepam. Thus, the amino acid residue β3Q64 on "loop" D clashed with the ester group of Ro15-1788. This explained the absence of a binding of this compound in the α1-plus/β3-minus pocket. In the case of flurazepam, the aromatic ring of β3Y62 on "loop" D exhibited a rotation different from that of γ2F77 on "loop" D, due to the interference of its hydroxyl group with the pending phenyl ring of flurazepam. In addition, the charged residue β3D43 on "loop" G exhibited unfavourable interactions with the pending phenyl ring, overall explaining the

drastically reduced potency and altered efficacy of flurazepam (Fig. 3.5 D). It thus seemed clear that the respective residues of the β3 subunit had to be replaced by the homologous residues of the γ2 subunit. This was done in the present study by generating the construct β3DGpCI.

In addition, the structural model predicted that β3G127 on "loop" E, could not form the H-bridge with the imidazo group of Ro15-1788. It was thus hypothesized that the introduction of an additional mutation into the β3 subunit converting the β3G127 to a threonine should increase the affinity and potency of Ro15-1788. Therefore the construct β3DGEpCI was generated that contained the first three mutations on loops D and G as in β3DGpCI, as well as the fourth mutation β3G127T.

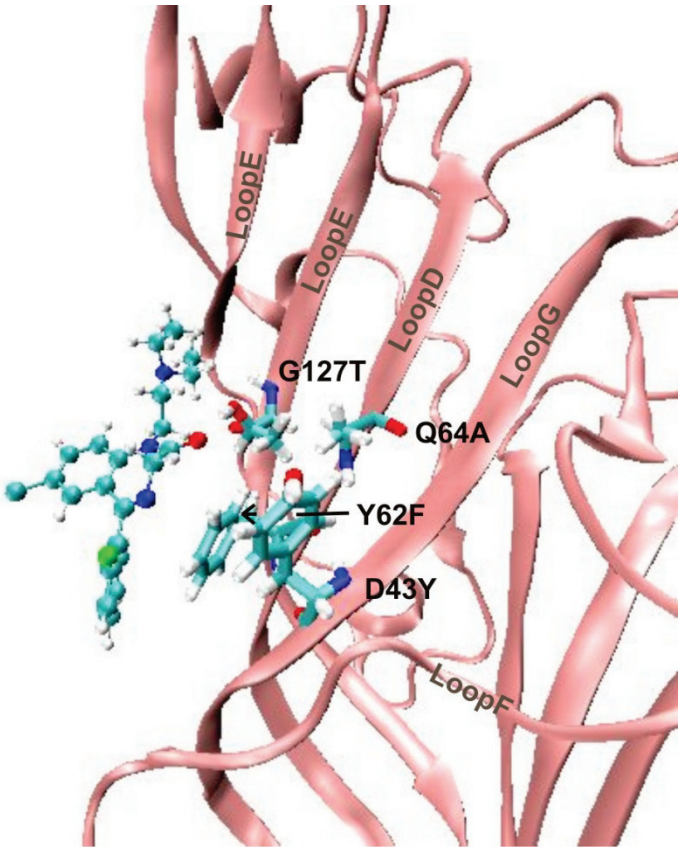


**Fig.4.1: Flurazepam bound model of the β3 subunit minus site, with the engineered benzodiazepine pocket lining interface of the α1β3DGE recombinant receptor. Flurazepam is shown in ball-and-stick representation, with light-blue C-C bonds, H atoms in white, O atoms in red, N-atoms in blue, F atoms in green. The protein is**

**shown in ribbon mode with red for the β3 subunit. Interacting side chains of the subunit are in stick representation in the same colour coding as flurazepam. Model developed by Richter (Richter submitted), modified picture provided by M. Ernst.**

## 4.2. Introduction of Four Point Mutations into the β3-Minus Side Successfully Incorporates a Benzodiazepine Binding Site in α1β3 Receptors

In addition to the constructs containing the triple (β3DGpCI) or quadruple mutations (β3DGEpCI), two other constructs were generated that contained either one (β3Y62F) mutation (β3D1pCI) or two mutations (β3Y62F and β3Q64A; β3D2pCI ) in "loop" D. These mutations seemed to be the minimum requirement for introducing a benzodiazepine binding at the α1-plus/β3-minus interface and should be ready, in case of success with the other two constructs containing three or four mutations. All of these constructs resulted in the expression of mutated β3 subunits that could assemble with α1 subunits and form receptors at the surface of HEK cells and Xenopus oocytes.

Finally, due to the unclear role of the γ2-"loop" F in the binding and function of benzodiazepines, two additional constructs were generated, in which a total of 7 or a total of 12 amino acid residues of the β3-"loop" F were exchanged for the corresponding γ2-"loop" F residues, respectively. Since these chimeric β3 constructs did not result in an adequate expression and/or assembly with α1 subunits, these constructs were not further investigated.

To increase our chances to rapidly obtain information on whether it is possible to introduce a benzodiazepine binding site into the α1-plus/β3-minus interface, in the present diploma work we only investigated the triple (α1β3DG) or quadruple mutated (α1β3DGE) recombinant receptors in pharmacological studies. For a complete characterization of the amino acid residues triggering allosteric action, positive modulation and potency, however, not only recombinant receptors α1β3DG, α1β3DGE, but also receptors α1β3D1 and α1β3D2, as well receptors containing additional mutations would have to be investigated individually.

Introduction of the three or the four point mutations into the β3 subunit converted the low potency, negative modulatory activity of flurazepam and the null modulatory activity of Ro15-1788 exerted in wild type α1β3 receptors, into a high potency positive modulation. As predicted by our structural model, the introduction of the fourth point mutation β3G127T increased the potency of Ro15-1788 (Fig. 3.6 D) for modulating GABA-induced chloride flux.

Although the potency of flurazepam in α1β3DGE receptors is still about 100 fold lower than that in wild-type α1β3γ2 receptors, its efficacy for enhancing GABA-induced chloride flux is about twice of that in wild-type receptors. This possibly could be explained by the fact that in α1β3DG and α1β3DGE receptors there are two sites with newly introduced benzodiazepine like binding properties (Fig. 1.4 C) in contrast to α1β3γ2 receptors that contain only the classical benzodiazepine binding site (Fig. 1.4 A). A simultaneous interaction of flurazepam with the two newly introduced benzodiazepine binding sites could thus explain the stronger efficacy of this compound in the mutated receptors.

Interestingly, Ro15-1788 exhibits a lower potency but a higher efficacy for receptors composed of α1β3DG than for those composed of α1β3DGE subunits. This possibly can be explained by a simultaneous low affinity interaction of Ro15-1788 with the two benzodiazepine binding site-like interfaces of α1β3DG receptors. In α1β3DGE receptors it then has to be assumed that Ro15-1788, although interacting with higher affinity with the two binding sites might exhibit a reduced efficacy for enhancing GABA-induced chloride flux. In addition, the flat dose response curve of Ro15-1788 for α1β3DGE receptors shown in Fig. 3.6 (D) seems to indicate that binding of Ro15-1788 to one of the mutated sites allosterically reduces binding to the other site.

Since Ro15-1788 exhibits a potency of approximately 100nM for enhancing GABA-induced chloride flux in α1β3DGE receptors, it was interesting to investigate whether a high affinity binding site for this compound could be demonstrated in these receptors. These receptors were therefore expressed in HEK cells and membranes from these cells were subjected to [$^3$H]Ro15-1788 binding studies in the absence or presence of 100μM diazepam or Ro15-1788. Due to the low amount of [$^3$H]Ro15-1788 specifically bound to α1β3DGE receptors the data were quite variable in three

different experiments. Nevertheless, Scatchard analysis of specific [$^3$H]Ro15-1788 binding to α1β3DGE receptors expressed in HEK cells indicated a high affinity binding with $K_D$ values of 14±5nM, 42±21nM and 63±22nM (mean±S.D.), that could not be identified with wild-type α1β3 subunits (experiments not shown). This demonstrates, that local determinants within the pocket lining interface are sufficient to induce positive modulation and high affinity binding by benzodiazepine site ligands in the β3 subunit of the $GABA_A$ receptor. Compared to α1β3γ2 receptors exerting a $K_D$ of 3.6nM, binding of α1β3DGE receptors is 5-20 times lower. The average $K_D$ of approximately 40nM, however, fits quite well to the estimated potency of this compound of approximately 100nM in electrophysiological experiments.

Similarly, the total number of binding sites identified (between 159-807fmoles/mg, depending on transfection efficiencies) in α1β3DGE receptors was less than 7.5% of that identified in α1β3γ2 receptors (10.280 fmoles/mg) determined in parallel experiments. Since in other experiments a similar expression of α1β3γ2 and α1β3DGE receptors was demonstrated at the cell surface, further experiments will have to determine whether binding to the newly introduced benzodiazepine binding site in α1β3DGE is labile and exhibits a high off rate, or whether only a low percentage of α1β3DGE receptors exhibits a conformation that allows binding of Ro15-1788 (Klausberger, Ehya et al. 2001).

Since a high affinity Ro15-1788 binding site could be introduced in α1β3DGE receptors, it was interesting to also study the interaction of flurazepam with this binding site. α1β3DGE receptors were thus expressed in HEK cells and specific binding of 10nM or 30nM [$^3$H]Ro15-1788 to membranes of these cells was inhibited by increasing concentrations of flurazepam. As shown in Fig. 3.8, flurazepam exhibited a $K_i$ of 7.1μM, that is approximately 100 fold larger than that for α1β3γ2 receptors ($K_i$ of 75nM), and fits nicely to the potency of flurazepam for enhancing GABA-induced chloride flux (approximately 10μM) in electrophysiological experiments (Fig. 3.5 D).

### 4.3. Conclusion and Outlook

Using predictions of our structural model of the benzodiazepine binding site of $GABA_A$ receptors we identified four out of 30 amino acid residues, that are different between the β3- and the γ2-minus side, as being important for the binding of benzodiazepines (for the alignment see appendix). Replacement of these four amino acid residues of the β3-minus side by the homologous residues of the γ2-minus side indeed converted a low potency negative modulatory activity of flurazepam and a null modulatory activity of Ro15-1788 in α1β3 receptors to a high potency positive modulatory effects of these compounds in the mutated α1β3DGE receptors, as predicted by the model. This supports previous conclusions that potency as well as efficacy seem to be mediated by the minus side of β3 subunits with γ-character (Richter submitted).

Although featuring these highly promising characteristics, the potency, affinity, and efficacy of these compounds at the mutated receptors is still not identical to that at the wild type α1β3γ2 receptors, indicating that the benzodiazepine binding site engineered into the α1-plus/β3-minus interface is still incomplete. Using information from a combination of modelling and experimental data, further mutations enhancing the interaction of the compounds with the β3-minus side can be proposed, that hopefully will allow to engineer a benzodiazepine binding site in α1β3 receptors that is more similar to that in wild type α1β3γ2 receptor.

Such mutations might also change the efficacy of Ro15-1788. This compound is a null modulator at the benzodiazepine binding site of $GABA_A$ receptors, but positively modulated the GABA currents of recombinant α1β3DG and α1β3DGE receptors. Such changes in the allosteric activity of Ro15-1788 were reported previously due to mutations in the γ subunit (Mihic, Whiting et al. 1994), leading to the conclusion that minor changes in the interaction of this compound with both α1 and γ2, or α1 and β3 subunits could change its efficacy.

The biological cause of the low potency but high efficacy of Ro15-1788 and flurazepam in α1β3DG receptors and high potency, low efficacy of Ro15-1788 in α1β3DGE receptors also is not clear. Although it was speculated above that this

could have been due to the presence of two engineered benzodiazepine binding sites in these mutated receptors, a functional coupling between some other binding sites might also have caused these effects and triggered structural rearrangements or changes in affinity (Kucken, Teissere et al. 2003). This question could be answered by generating α1β3DGEγ1 receptors. γ1 receptors were reported to be insensitive to Ro15-1788 and to exert a low affinity for flurazepam (Benke, Honer et al. 1996; Khom, Baburin et al. 2006). The co-expression of a γ1 together with α1 and recombinant β3DG or β3DGE subunits will result in only one engineered α1-plus/β3-minus benzodiazepine binding site in these receptors that can be modulated by Ro15-1788 and would make it possibe to investigate the pharmacological effect of a single engineered benzodiazepine binding site.

Finally, the low number of incorporated [$^3$H]Ro15-1788 binding sites in HEK cells transfected with α1β3DGE receptors could either be explained by a low number of binding sites formed or by a high off rate of [$^3$H]Ro15-1788. In future experiments this question can be answered by [$^3$H]EBOB ([$^3$H]ethynylpropylbicycloorthobenzoate) binding studies. This compound is an open channel blocker of $GABA_A$ receptors probably binding directly into the chloride ion channel of these receptors (Atucha, Hammerschmidt et al. 2009). Using [$^3$H]EBOB binding we thus can determine the total number of receptors formed. In case the number of α1β3γ2 receptors formed in HEK cells is comparable with that of α1β3DGE receptors, as suggested by experiment investigating cell surface expression of α1β3γ2, α1β3, and mutated receptors (Fig. 3.3. B), the off-rate of [$^3$H]Ro15-1788 binding can be directly compared in these receptors by adding high concentrations of Ro15-1788 and measuring the radioactivity remaining on the membranes at different time points.

Thus, although we have established a feasible and highly interesting α1β3 model system with an engineered high affinity benzodiazepine binding site, there are still several effects we cannot fully explain. Further experiments will have to be performed to answer all open questions.

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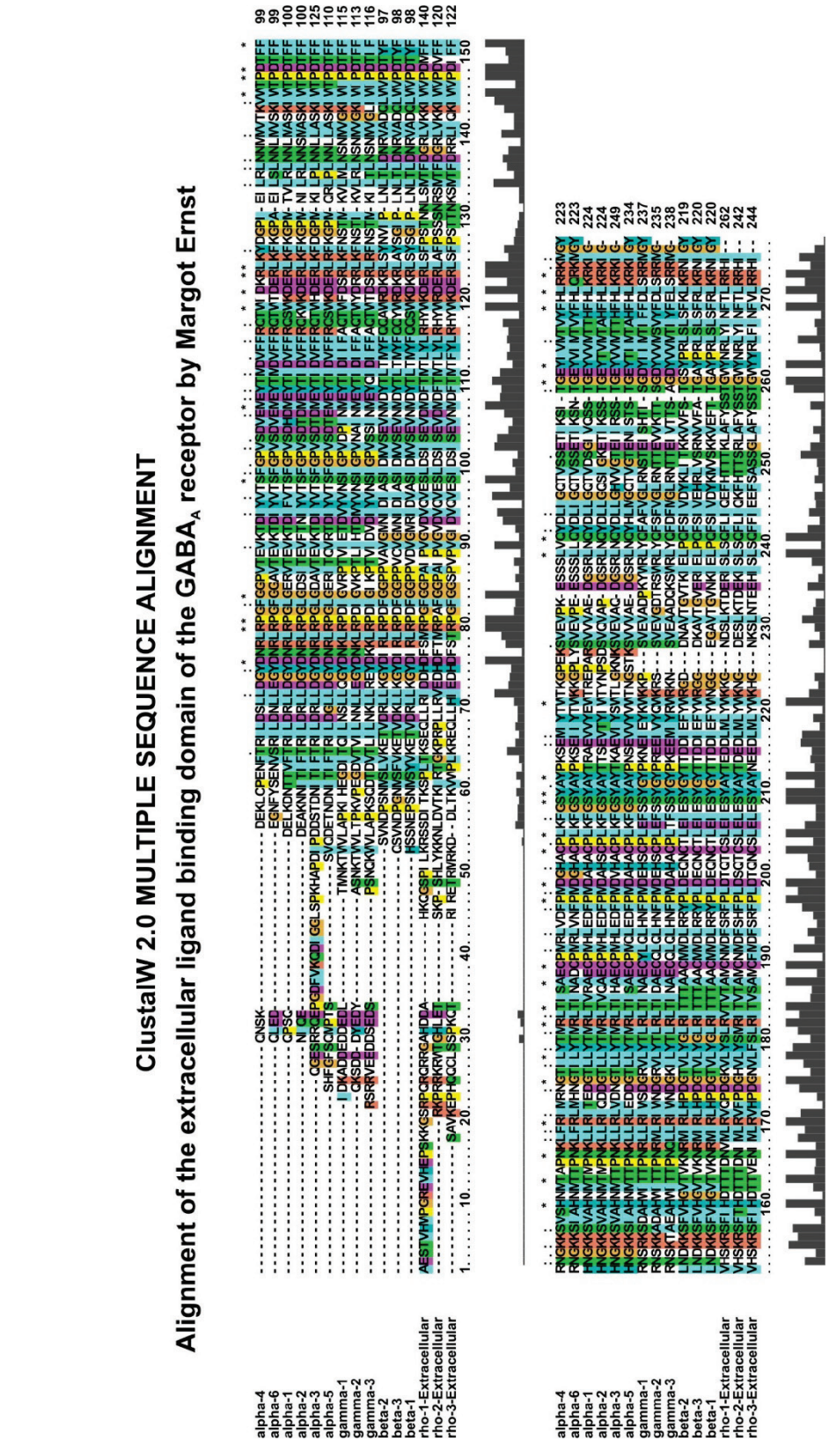
# 6. APPENDIX


ClustalW 2.0 MULTIPLE SEQUENCE ALIGNMENT
Alignment of the extracellular ligand binding domain of the GABA$_A$ receptor by Margot Ernst
alpha-4
alpha-6
alpha-1
alpha-2
alpha-3
alpha-5
gamma-1
gamma-2
gamma-3
beta-2
beta-3
beta-1
rho-1-Extracellular
rho-2-Extracellular
rho-3-Extracellular

# 7. CURRICULUM VITAE

**PERSONAL DETAILS:** Name: **Anna Lidia Deréky**

Date of birth: **16th August 1985, Vienna, Austria**

Nationality: **Austrian**

**EDUCATION:**

| | |
|---|---|
| 2009-2010 | Master-Thesis work at the Center of Brain Research in the group of Prof. Sieghart, Department of Biochemistry and Molecular Biology |
| 2009 Vienna | Merit grant for excellent academic achievements from the University of |
| 2007-2008 Kingdom | One-year ERASMUS scholarship at the University of Glasgow, United |
| 2007 Hungary | Three month long CEEPUS research fellowship at the University of Pécs, |
| 2008 Biology | Graduation from the University of Vienna with a BSc degree in Molecular |
| 1996-2003 | Realgymnasium Rahlgasse, Matura (A-level) with distinction on 12th of June 2003, Vienna |
| 1995-1996 | Central European School (Secondary School), Budapest |
| 1991-1994 | Elementary School Aspernallee, Vienna |

**PROFESSIONAL EXPERIENCE:**

| | |
|---|---|
| September 2009 | Austrian Neuroscience Association conference poster presentation, Salzburg |
| September 2008 | Neurobiology Summer School: "From receptor cells to cognition" participation, Vienna |
| July 2008 | FENS conference participation, Geneva |

**LANGUAGE SKILLS:**

German (mother tongue)

Hungarian (mother tongue)

English (TOEFL ibT score: 104)

French (DELF Level B2)

April 2010, Vienna Anna Lidia Deréky