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Recombinant GABA_A receptor expression in the
baculovirus expression system

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1 INTRODUCTION

1.1 The neurotransmitter γ -aminobutyric acid

The γ -aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the mammalian central nervous system (CNS). Approximately 20-50% of all central synapses are supposed to use GABA as a transmitter, depending on the brain region (Bloom and Iversen 1971; Young and Chu 1990; Sivillotti and Nistri 1991; Halasy and Somogyi 1993).

GABA is formed in vivo by a metabolic pathway referred to as the GABA shunt. The GABA shunt is a closed-loop process with the dual purpose of producing and conserving the supply of GABA. Glucose is the principal precursor for GABA production in vivo, although pyruvate and other amino acids also can act as precursors (Fig. 1).

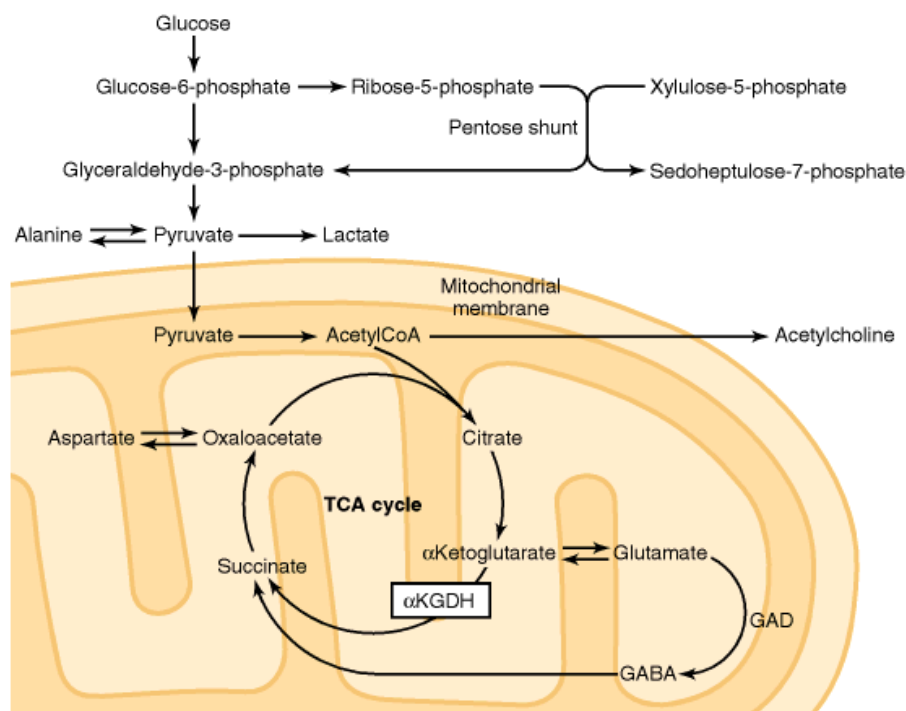


Fig. 1: Simplified schematic diagram of the major metabolic pathways linking glycolysis and tricarboxylic acid cycle (TCA or Krebs cycle) flux to the synthesis of neurotransmitters (Siegel et al. 1999).

The first step in the GABA shunt is the transamination of α -ketoglutarate, a Krebs cycle intermediate, to glutamic acid. Then the glutamic acid decarboxylase

(GAD) catalyses the decarboxylation of glutamic acid to form GABA (Awapara et al. 1950; Roberts and Frankel 1950). The glutamic acid decarboxylase appears to be expressed only in cells that use GABA as a neurotransmitter (Siegel et al. 1999). GABA can then be metabolized by transamination to succinic semialdehyde, and further oxidized to succinic acid, which can re-enter the Krebs cycle (Fig. 2).

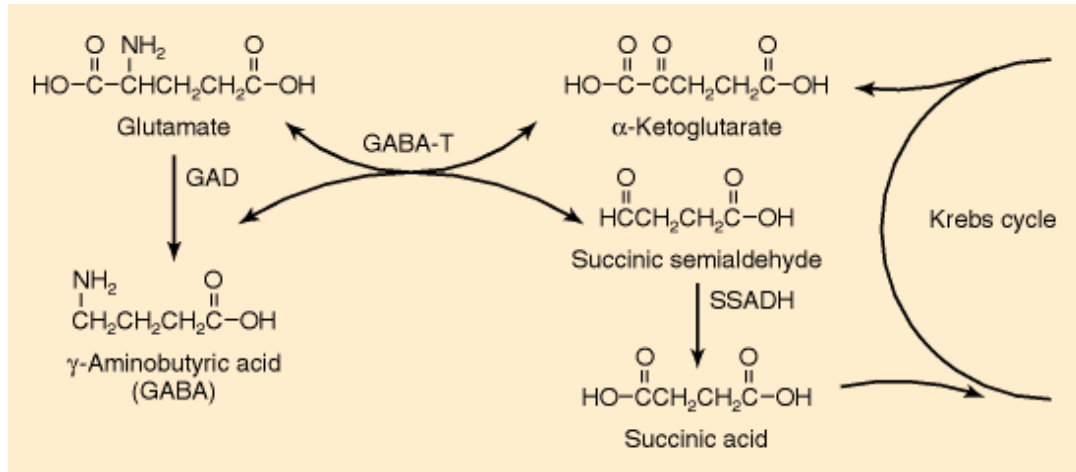


Fig. 2: GABA shunt reactions are responsible for the synthesis, conservation and metabolism of GABA (Siegel et al. 1999).

GABA is stored in vesicles close to the synaptic membrane and is released into the synaptic cleft in response to a Ca^{2+} dependent process after depolarization of the presynaptic membrane. In the synaptic cleft GABA affects receptors on the postsynaptic membrane. The re-uptake by presynaptic terminals or glia cells occurs via highly specific transmembrane transporters (Minelli et al. 1995) (Fig. 3).

The neurotransmitter GABA acts by two different classes of receptors, the GABA_A and the GABA_B receptors (Hill and Bowery 1981; Bowery et al. 1983; Bowery 1993). GABA_A receptors are Cl^- channels and are stimulated by GABA, muscimol, and isoguvacine, and are inhibited either competitively by bicuculline or non-competitively by picrotoxin and TBPS (t-butylbicyclophosphorothionate) (Macdonald and Olsen 1994) (Fig. 4). GABA_B receptors are stimulated by GABA and (-)baclofen and are inhibited by phaclofen (Hill and Bowery 1981). The latter receptors seem to be coupled indirectly via an intervening G-protein or a second messenger system to Ca^{2+} and K^+ channels (Bormann 1988; Bowery 1993) (Fig. 4).

Other GABA receptors, the GABA_C receptors, are stimulated by GABA and certain conformationally restricted analogues of GABA, such as cis-4-aminocrotonic acid (CACA). This class of receptors is insensitive to both bicuculline and (-)baclofen (Johnston 1994; Bormann and Feigenspan 1995; Johnston 1996). Since these

receptors are formed by ρ subunits that are homologous to GABA_A receptor subunits it was decided that they should be classified as a subgroup of GABA_A receptors (Barnard et al. 1998).

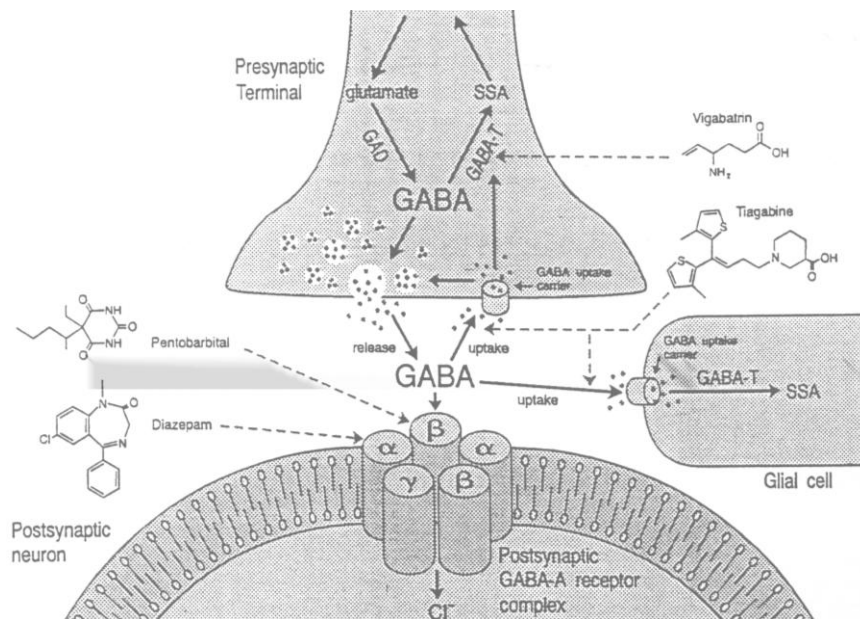


Fig. 3: Schematic representation of a GABAergic synapse (Suzdak and Jansen 1995).

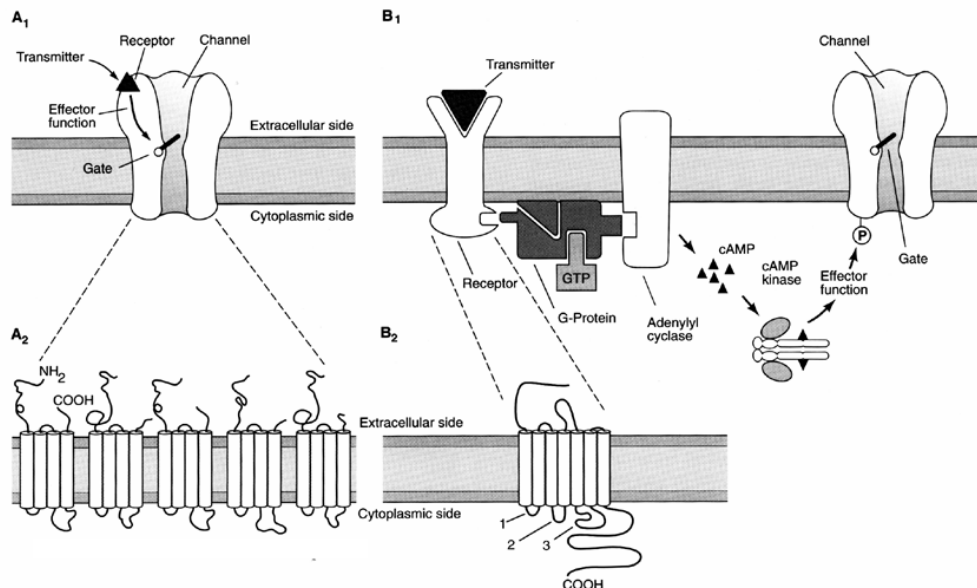


Fig. 4: GABA_A and GABA_B receptor types. Direct gating of ionotropic GABA_A receptors (A₁, A₂). Indirect gating of metabotropic GABA_B receptors. This is mediated by a second messenger that couples the GABA_B receptor to the ion channel (B₁, B₂). The transmembrane structure of GABA_A or GABA_B receptor subunits is indicated in A₂ or B₂, respectively (Kandel et al. 1991).

1.2 Pharmacology of GABA_A receptors

GABA_A receptors are ligand gated ion channels that can be opened by GABA and are the major inhibitory neurotransmitter receptors in the CNS (Macdonald and Olsen 1994). If GABA binds to the GABA_A receptor, the neuronal membrane conductance for chloride ions increases. In most cases the intracellular chloride ion concentration is low and the extracellular concentration is high. Therefore the chloride gradient across the membrane forces chloride into the cell.

At the resting membrane potential, however, this effect is more or less balanced by the electrochemical driving force that inhibits chloride entry because of the negative charge inside of the cell. Opening of chloride ion channels in unexcited neurons, thus, usually results in a slight membrane hyperpolarization and in a reduced neuronal excitability of the cells, because the increased chloride ion conductance counteracts the effects of depolarizing stimuli (Bormann 1988).

In some cases, however, excitatory actions of GABA have been demonstrated. This unusual effect was demonstrated in developing brain tissue (Cherubini et al. 1991), in some neurons from adult brain (Avoli 1992), in astrocytes and oligodendrocytes (von Blankenfeld and Kettenmann 1991), generally in cells possessing a high intracellular chloride ion concentration. The chloride gradient across the membrane of the respective cells is thus much smaller than in cells where GABA exerts a hyperpolarizing action. On opening of chloride ion channels, the electrochemical driving force pushes chloride ions out of the cell and this results in a depolarization of the membrane potential.

Not only GABA is able to bind and thereby modulate the GABA_A receptor but also many different drugs. Thus, benzodiazepines, barbiturates, steroids, anaesthetics, convulsants, or drugs that cannot be summarized into groups, like loreclezole (Sieghart 1995), modulate the action of GABA by interacting with distinct allosteric binding sites on GABA_A receptors.

Thus binding studies and electrophysiological and behavioral experiments indicate that the anxiolytic, anticonvulsant, muscle relaxant and sedative-hypnotic benzodiazepines, and some depressant barbiturates enhance the action of GABA on GABA_A receptors. In contrast, some anxiogenic or convulsant β -carbolines, the convulsants bicuculline, picrotoxin, or t-butylbicyclophosphorothionate (TBPS) reduce the actions of GABA on this receptor. Binding of such substances induces a conformational change in the GABA_A receptors that in turn influences the binding

properties of other binding sites present on these receptors (causing complex allosteric interactions of these binding sites) and modulates GABA-induced chloride ion fluxes (Sieghart 1995).

Based on their pharmacological action it was concluded that GABA_A receptors are involved in controlling the excitability of the brain (Olsen and Avoli 1997; Fritschy et al. 1999), in the modulation of anxiety (Nutt 1990; Pratt 1992), of feeding and drinking behaviour (Cooper 1989), circadian rhythms (Turek and Van Reeth 1988), cognition, vigilance, memory and learning (Sarter et al. 1988; Izquierdo and Medina 1991).

1.3 Molecular structure of GABA_A receptors

GABA_A receptors are hetero-oligomeric receptors belonging to the super-family of ligand gated ion channels (Schofield et al. 1987), like the nicotinic acetylcholine (nACh), the glycine and the 5-HT₃ receptors (Betz 1990; Ortells and Lunt 1995). The receptor is a pentamer (Nayeem et al. 1994; Tretter et al. 1997) and each of the five subunits comprises a large extracellular N-terminal domain, four transmembrane (TM) domains and a large intracellular loop between TM3 and TM4. The second transmembrane domain (TM2) is gating the ion channel pore (Fig. 5).

So far six α , three β , three γ , one δ , one ϵ , one π , one θ , and three ρ subunits have been cloned and sequenced from the mammalian brain (Barnard et al. 1998; Bonnert et al. 1999). Immunohistochemical studies showed a distinct and often widespread distribution for each GABA_A receptor subunit throughout the brain (Fritschy et al. 1998; Pirker et al. 2000). The resulting expression of multiple subunits in the same neurons suggests the existence of a large variety of GABA_A receptors in the brain. Subunits, however, seem to combine only in specific combinations determining both the pharmacological and the electrophysiological property of this respective GABA_A receptor (Sieghart 2000).

Recently, a subtractive purification method was established, which allows the determination of the subunit composition of the native GABA_A receptor subtypes (Jechlinger et al. 1998; Bencsits et al. 1999). Combined with studies investigating the subunit stoichiometry (Tretter et al. 1997), results indicated that the majority of GABA_A receptors comprise two α , two β and one γ subunit (Sieghart et al. 1999) (Fig. 6).

Investigations indicate that the γ subunit can be substituted by the δ , ϵ , or π subunit (Shivers et al. 1989; Hedblom and Kirkness 1997; Whiting et al. 1997) and the

β subunit by the θ one (Whiting 1999) (Fig. 6). In contrast the ρ subunits seem to form only homo- or hetero-oligomeric receptors with other ρ subunits, forming the previously described “GABA_C” receptors (Johnston 1994; Bormann and Feigenspan 1995; Johnston 1996).

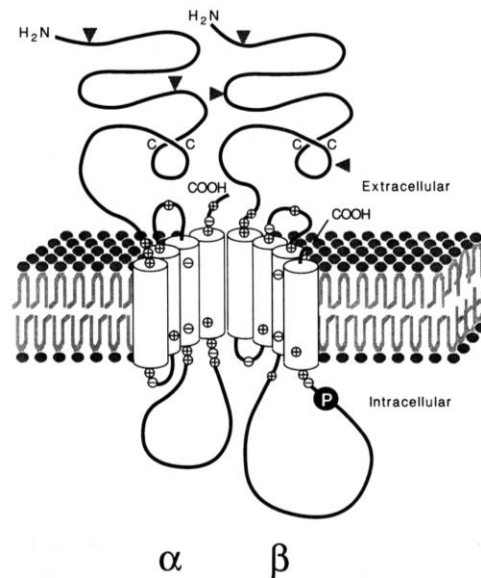


Fig. 5: Schematic model for the topology of the GABA_A receptor in the cell membrane. Four transmembrane domains in each subunit are shown as cylinders. The structure in the extracellular domain is drawn in an arbitrary manner and shows the cys-loop formed by the disulfide bond predicted at the conserved cysteines. Potential extracellular sites for N-glycosylation are indicated by triangles. Those charged residues, which are located within or close to the ends of the membrane domains, are indicated in small circles with charge marked. An encircled P marks the site for cAMP-dependent serine phosphorylation, present only in the β subunit (Schofield et al. 1987).

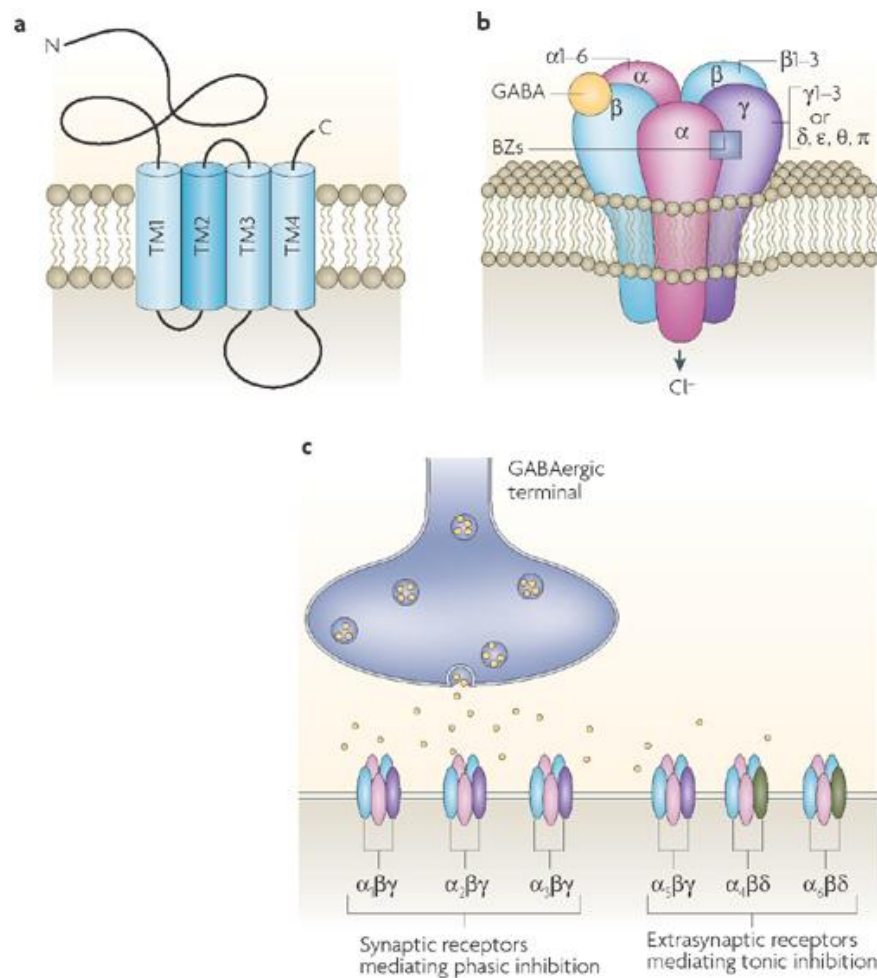


Fig. 6: Molecular structure of GABA_A receptors (Jacob et al. 2008).

1.4 Trafficking of GABA_A receptors

GABA_A receptors subunits are synthesized and assembled in the endoplasmic reticulum (ER). These processes can be modulated by ubiquitylation and subsequent ER-associated degradation by the proteasome. Ubiquitylated GABA_AR subunits can also be modulated by their association with PLIC1. PLIC1 facilitates GABA_AR accumulation at the synapse by preventing the degradation of ubiquitylated GABA_ARs. The traffic into the Golgi network and subsequent trafficking to the plasma membrane are also facilitated by a number of GABA_AR-associated proteins (GABARAP). These proteins associate with the $\gamma 2$ subunit. N-ethylmaleimide-sensitive factor (NSF) and brefeldin-A-inhibited GDP/GTP exchange factor 2 (BIG2) bind to the β subunits and modulate GABA_AR trafficking in the Golgi. There the palmitoylation of γ subunits takes place as a result of an association with the palmitoyltransferase Golgi-specific DHHC zinc-finger-domain protein (GODZ), and it is a critical step in the delivery of GABA_ARs

to the plasma membrane. GABA_AR-interacting factor proteins (GRIFs) and Phospholipase-C-related catalytically inactive proteins (PRIPs) play an important role during the trafficking of GABA_ARs to the membrane and the phosphorylation state of GABA_ARs (Chen et al. 2007; Jacob et al. 2008) (Fig. 7).

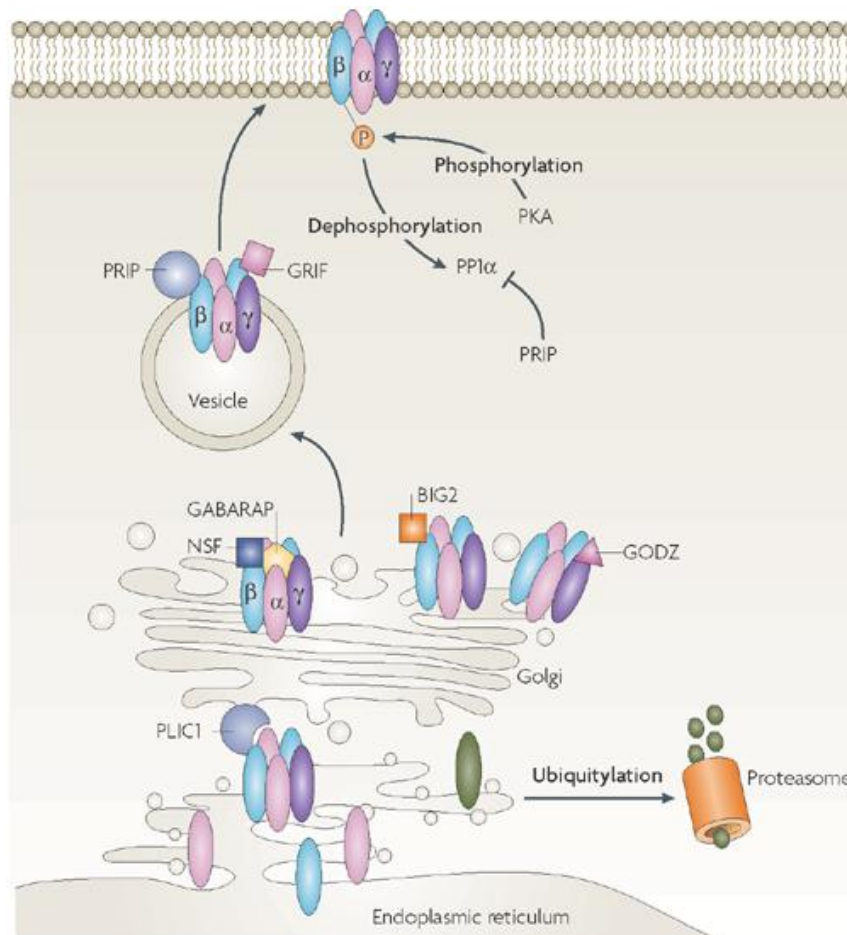


Fig. 7: GABA_A receptor trafficking in the cell (Jacob et al. 2008).

1.5 Recombinant protein expression systems

In basic research and in terms of large-scale production of recombinant proteins the expression of foreign genes in diverse host organisms has become particularly interesting. The usage of classical approaches with yeast and *E. coli* as expression hosts has been extended by insect cell and mammalian cell expression systems in the last years.

The insect cell and mammalian cell expression systems show huge advantages compared to yeast and *E. coli*. Expressed recombinant proteins show essential posttranslational modifications (PTM) such as glycosylation (by cell specific enzymes

like glucosidases, mannosidases and N-acetylglucosamine transferases), phosphorylations, carboxylation and fatty acid acylation. In addition there is a controlled signal and proteolytic cleavage, which are necessary for proper folding and function of foreign proteins. Tab. 1 shows a comparison of the different recombinant protein expression systems.

Characteristics	E. coli	Yeast	Insect cells	Mammalian cells
Cell Growth	Rapid (30 Min)	Rapid (90 Min)	Slow (18-24 H)	Slow (24 H)
Complexity of Growth Medium	Minimum	Minimum	Complex	Complex
Cost of Growth Medium	Low	Low	High	High
Expression Level	High	Low - High	Low - High	Low - Moderate
Extracellular Expression	Secretion to Periplasm	Secretion to Medium	Secretion to Medium	Secretion to Medium
Protein Folding	Refolding Usually Required	Refolding May Be Required	Proper Folding	Proper Folding
N-linked Glycosylation	None	High Mannose	Simple, No Sialic Acid	Complex
O-linked Glycosylation	No	Yes	Yes	Yes
Phosphorylation	No	Yes	Yes	Yes
Acetylation	No	Yes	Yes	Yes
Acylation	No	Yes	Yes	Yes
gamma-Carboxylation	No	No	No	Yes
Yield (mg) (per liter culture)	50-500	10-200	10-200	0.1-100
Success Rate (%) (soluble or functional)	40-60	50-70	50-70	80-95
Project Cost	Low	Low	Middle	High
Recommended Use	Antigen protein, Protein standards, Functional proteins	Proteins with glycosylation, Vaccine, Secreted form, Alternative to insect cell system	Proteins with glycosylation, Assay standards, Secreted form, Alternative to yeast system	Functional study, PTM study, Assay standards, Characterization
Advantage	Simple, robust, lowest cost, highest yield	Simple, low cost, good for certain proteins	Relatively higher yield, better PTM	Natural protein configuration, best PTM
Disadvantage	Least PTM ^a	Longer time, less PTM	Longer time, higher cost	Highest cost, lower yield

Tab. 1: Comparison of different recombinant protein expression systems. PTM = posttranslational modifications (<http://www.genwaybio.com>).

1.6 Baculovirus expression vector system

Over the past 25 years the baculovirus-insect cell expression system has become one of the most widely used systems for routine production of recombinant proteins. A number of technological improvements have eliminated the original tedious procedures required to identify and isolate recombinant viruses, increasing the popularity of the system. These include development of a wide variety of transfer vectors, simplified recombinant virus isolation and quantification methods, advances in cell culture technology and the commercial availability of reagents. These

enhancements have resulted in a virus-based expression system that is safe, easy to use and readily amenable to scale-up (Kost et al. 2005; Jarvis 2009).

Baculoviruses make up a family of viruses and are grouped into nuclear polyhedrosis viruses (NPV) and granulosis viruses. More than 500 different types of baculoviruses have been discovered and the host range is restricted to invertebrates, mostly to insects (eg, moths and butterflies)(Hu 2005).

Among the numerous baculoviruses, *Autographa californica* multiple NPV (AcMNPV) is the most well studied and most extensively used. AcMNPV has a circular double-stranded DNA genome of approximately 130kbp (Blissard and Rohrmann 1990).

The DNA is condensed with a protamine –like protein into the core and packed into the nucleocapsides. Nucleocapsides are synthesized in the nucleus of infected cells (typically 40nm-50nm in diameter and 200nm-400nm in length). Membrane-enveloped Nucleocapsides are referred to as virus particles or virions. In nature, AcMNPV are occluded in a polyhedron (2µm-15µm in size) mainly consisting of polyhedron protein. After ingestion by insects, the polyhedron matrix is dissolved in the alkaline midgut, thus releasing the embedded virions, which subsequently infect epithelial cells of the intestine. Early in the infection cycle, the DNA genome is replicated and transcribed in the nucleus and the nucleocapsides are assembled. (Hu 2005).

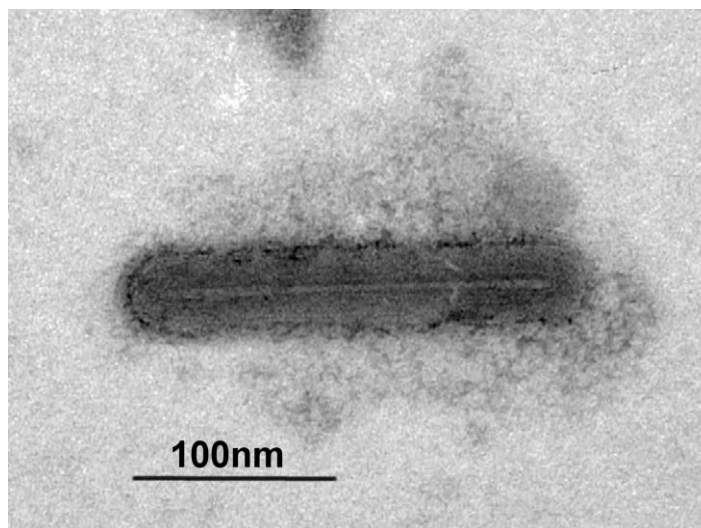


Fig. 8: Electron micrograph of a baculovirus

(<http://www.mardre.com/homepage/mic/tem/samples/bio/virus/bac2.htm>).

In the infected cell the expression of polyhedron protein is confined to the late stages of the infection cycle (15-96h post-infection), after production of the virus

particles which spread the infection to other cells. The polyhedron protein accumulates to very high levels within the infected cell and has been variously estimated to account for 20-50% of the total cell associated protein by the end of the virus replication cycle. Polyhedrin gene deletion mutants of AcMNPV produce infectious virus particles but not polyhedron inclusion bodies. This feature of the virus allowed the substitution of foreign genes in place of the polyhedron and their expression to high levels in cells infected with the recombinant viruses (Possee and Howard 1987). Viral budding and infection of new cells through adsorptive endocytosis are mainly triggered by the virus-coded glycoprotein gp64 which plays a key role in the baculoviral life cycle. This protein forms trimers and accumulates at the poles of the capsid giving it its characteristic morphology (Whitford et al. 1989).

Several hundred different genes of viral, animal and herbal origin have already been expressed successfully using the baculovirus expression system. Expression yields can reach as much as 500mg per litre culture volume taking advantage of the very strong polyhedron and p10 promoters. Moreover, the separation of virus production and foreign gene expression with these very late promoters facilitates the production of possibly cytotoxic proteins as shown by Baum et al. (Baum et al. 1987).

The most commonly used insect cell lines are the Sf9 and the Sf21 cell lines. They both originated from IPLB (USDA Insect Pathology Laboratory at Beltsville, Maryland). They are derived from *Spodoptera frugiperda* pupal ovarian tissue. They grow at a doubling time of approximately 18-22 hours in both, monolayer and suspension culture. Cells can be propagated in serum-supplemented media as well as in serum-free media (Vaughn et al. 1977).

Insect cells are capable of performing many of the posttranslational modifications necessary for the production of functionally active proteins. It is equally true that insect protein processing pathways are not necessarily equivalent to those of higher eukaryotes. Most baculovirus-expressed recombinant glycoproteins will acquire authentic N-glycans only at sites occupied by high mannose structures on the native mammalian products. In contrast, they are most likely acquired paucimannose N-glycans at sites occupied by complex, terminally galactosylated and/or sialylated N-glycans on the native product (Fig. 9) (Kost et al. 2005).

All the advantages and the high working safety make the baculovirus expression system to a valuable and recommendable tool for the production of complex proteins, especially for applications in biomolecular research and medicine (Massotte 2003).

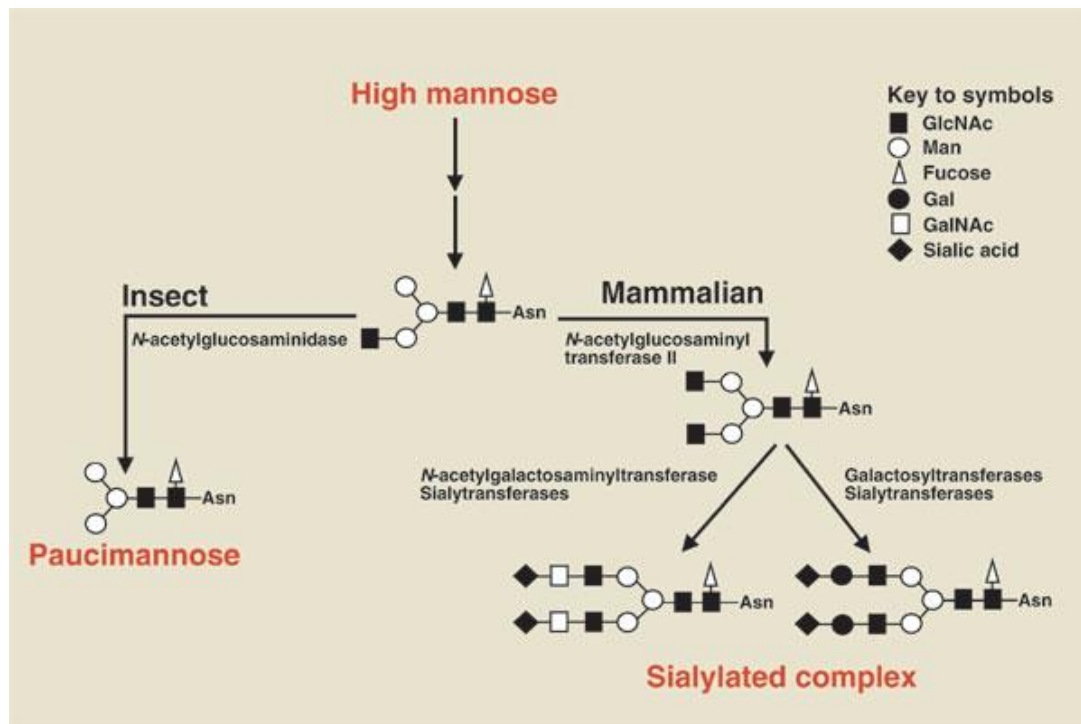


Fig. 9: Overview of processing pathways and major *N*-glycans produced by insect and mammalian cell systems. The processing pathways in both systems yield common intermediate. The major insect-cell end product (paucimannose) is produced by further trimming of this intermediate (left-hand branch), whereas the major mammalian-cell and products (including sialyted complex) are produced by elongation of this intermediate (right-hand branch) (Kost et al. 2005).

1.7 Scope of work

GABA_A receptors are targets for many clinically important drugs (Sieghart 1995) that influence excitability of the brain, motor function, anxiety, cognition, vigilance and memory. The exact molecular structure of the GABA_A receptor is still unknown, as it is the case for most receptors of the cys-loop receptor super-family.

The structural analysis of GABA_A receptors by crystallization or NMR techniques requires high amounts of pure receptors. The aim of this thesis is to produce recombinant hetero-oligomeric (α1-β3-γ2) GABA_A receptors. These receptors will be expressed in the baculovirus expression system, characterized and then further investigated together with collaborating international groups.

This thesis is based on the thesis “High yield expression and His-tag purification of recombinant GABA_A receptors using the Sf9-baculovirus Expression Vector System” from Leila Wabenegger, 2005. Produced and characterized virus stocks from the latter thesis were taken to produce new virus stocks or to start other expression experiments.

2 MATERIALS AND METHODS

2.1 Cloning

2.1.1 Plasmid DNA preparation

2.1.1.1 DNA Miniprep

For the purification of small amounts of DNA the QIAprep® Miniprep Kit (QIAGEN) was used. Therefore 20ml of LB-medium, containing 100µg/ml ampicillin, were inoculated with a single colony of *Escherichia coli* XL-1-Blue cells (Stratagene) transformed with a pCI, pUC18 or a pBAC 4x-1 vector. The cells were grown over night and 5ml of the culture were harvested.

LB-medium

10g Bactotryptone
5g Yeast extract
5g NaCl
ddH₂O to a total volume of 1000ml

QIAprep® Miniprep Kit (QIAGEN) buffers

P1	50mM Tris-Cl, pH 8.0 10mM EDTA 100µg/ml RNase A
P2	200mM NaOH 1% SDS (w/v)
N3	QIAGEN®
PE	QIAGEN®
EB	10mM Tris-HCl pH 8.5

The pellet was resuspended in 250µl buffer P1, transferred into a sterile 1.5ml Eppendorf tube and 250µl of lysis buffer P2 were added. The tube was inverted gently

4-6 times and incubated for 5min at room temperature. After addition of 350µl buffer N3 the tube was inverted 4-6 times and centrifuged for 10min at 15,700 x g.

The clear supernatant was applied to a QIAprep® spin column and centrifuged for 1min 15,700 x g at room temperature. The column was washed with 750µl buffer PE and centrifuged twice at 15,700 x g for 1 min. Then the column was transferred to a 1.5ml Eppendorf tube. The DNA was eluted with 75µl buffer EB (centrifugation: 1min, 15,700 x g, room temperature) and was stored at 4°C.

2.1.1.2 DNA Maxiprep (Endofree)

For subsequent transfection of Sf9 cells, plasmid DNA of high purity is essential. Therefore, the DNA was purified using the Endofree Plasmid® Maxi Kit (QIAGEN) according to the kit manual.

5ml of LB-medium containing 50µg/ml ampicillin were inoculated with a single colony and grown in a shaking incubator for 6 hours at 37°C. The starter culture was diluted with 400ml LB-medium including 50µg/ml ampicillin. The cells were grown at 37°C for 12-16 hours and harvested at an OD₆₀₀ of 1.5 - 1.8 (centrifugation: 15min, 4,600 x g, 4°C). The dry pellet was stored at -20°C until usage.

After thawing, the pellet was resuspended in 20ml buffer P1 (4°C). Then 20ml of buffer P2 were added, the solution was mixed gently and incubated for 5min at room temperature. After adding 20ml cold buffer P3, the lysate was incubated for 10min at 4°C and centrifuged (30min, 48,000 x g, 4°C). The clear supernatant (lysate) was transferred to a fresh tube, 5ml of ER buffer were added and the solution was incubated for 30min at 4°C.

A QIAGEN-tip 100 was equilibrated with 4ml buffer QBT and the lysate applied to it. The column was washed twice with 10ml buffer QC and the DNA was eluted with 5ml buffer QN. The DNA was precipitated with 3.5ml Isopropanol and centrifuged for 60min at 4,600 x g 4°C. The DNA pellet was washed with 2ml endotoxin-free 70% ethanol and centrifuged again (60min, 4,600 x g, 4°C). The DNA pellet was air dried and redissolved in 200µl buffer TE for 15min at 65°C. The DNA was then stored at 4°C.

2.1.2 Determination of DNA concentration by optical density measurement

The DNA concentration and quality were determined by measuring with a UV spectrometer (Hitachi U-2001).

450µl of a 1:30 dilution in ddH₂O of the DNA sample were prepared and transferred into a quartz cuvette. Double-distilled water was measured as a reference to subtract the background absorbance from the sample data. The concentration was calculated as described below:

$$C \text{ (}\mu\text{g/ml)} = \text{OD}_{260} \times \text{dilution} \times 50\mu\text{g/ml}$$

The DNA purity was estimated by the ratio between OD₂₆₀ and OD₂₈₀

2.1.3 DNA digestion with restriction enzymes

The digestion of DNA with restriction enzymes is necessary to prepare vector and insert for the ligation step. Enzymes and 10x reaction buffers were supplied from Roche Diagnostics and Promega.

The DNA was incubated with restriction endonucleases in the appropriate buffer at the optimum temperature for the enzymes for 1-16 hours. To purify the DNA fragments, an agarose gel electrophoresis was performed and the fragments were cut out under UV illumination. Vectors and digested PCR fragments were purified by using the GFX PCR and Gel Band Purification Kit (2.1.5.2).

2.1.4 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA by their size. The agarose concentration of the gel was chosen depending of expected length of DNA fragments:

1.0-10.0kbp: 0.8-1.0% Agarose

0.2-2.0kbp: 1.5-2.5% Agarose

For a 1% mini-gel (10x6.5cm), 0.5g agarose (Life Technologies) were added to 50ml 1xTBE buffer and heated in a microwave. After cooling to 50-55°C, 2.5µl ethidiumbromide (10mg/ml) were added. The gel was poured and was allowed to form completely at 4°C. The samples, mixed with 10x loading buffer, were loaded into the wells. After loading a DNA ladder, the gel was run at 8-10 V/cm. The DNA fragments were visualized under UV light and photographed.

10x TBE buffer

900mM Tris
900mM Boric acid
25mM EDTA

10x Loading buffer

7.5g Ficoll
0.01g Xylencyanol EF42
2ml 0.5M EDTA
pH 8.0
total volume of 25ml with MilliQ

2.1.5 Purification of DNA fragments

DNA fragments from PCR or agarose gel fragments were purified with the GFX PCR DNA and Gel Purification Kit from GE Healthcare (capture buffer, washing buffer, EB buffer). Using this method, primer, salts and other contaminations were eliminated quantitatively.

2.1.5.1 Purification of DNA fragments in solutions

A maximum of 100µl sample was mixed with 500µl capture buffer, applied on a GFX Microspin™ column, incubated for 1min and centrifuged at 15,700 x g for 30sec. The column was washed with 500µl washing buffer and centrifuged for 90sec at 15,700 x g. Then the column was transferred to a 1.5ml Eppendorf tube. To elute the DNA 15-50µl elution buffer EB were dropped on the filter in the column, incubated for 60sec at room temperature and centrifuged for 60sec at 15,700 x g.

2.1.5.2 Purification of DNA fragments from agarose gel

For every 100mg of agarose gel slice 100µl capture buffer were added and incubated at 60°C in a Thermomixer until the agarose was dissolved completely. The mixture was loaded onto a GFX Microspin column. The column was centrifuged, washed and DNA eluted as described in 2.1.5.1.

2.1.6 Ligation of DNA

For the ligation of a vector and an insert the Rapid DNA ligation Kit (Roche) was used. A molar ratio of the vector DNA to insert DNA should be between 1:1 to 1:5.

Ligation mix

2-4ng Vector DNA
10ng Insert DNA
1µl 5x DNA dilution buffer (Roche)
5µl 2x DNA ligation buffer (Roche)
3U T4 DNA ligase (Roche)
ddH₂O to 10µl total volume

The reaction mix was incubated for 30min at room temperature and used immediately for transformation (2.1.7).

2.1.7 Transformation

2.1.7.1 XL1-Blue competent cells

Competent *Escherichia coli* XL1-Blue cells were prepared in our lab and aliquots were frozen at -80°C. For transformation 100µl of XL1-Blue competent cells were thawed at room temperature, mixed with 8µl of the ligation reaction (see 2.1.6) and were incubated on ice for 30min. After heat shock (90sec at 42°C, water bath), the cells were incubated for 2min on ice and 400µl of SOC, pre-warmed to 42°C, were added. Then this suspension was incubated for 60min at 37°C and 50-200µl were plated on LB-plates with 50 or 100µg/ml ampicillin, respectively. The plates were incubated over night at 37°C.

SOB medium

10g Bactotryptone
2.5g Yeast extract
290mg NaCl
95mg KCl
dH₂O to a total volume of 500ml
Sterilized by autoclaving
5ml 2M MgCl₂ (sterile) were added before use

SOC medium

10ml SOB medium
100µl 2M Glucose (sterile)

2.1.7.2 XL1-Blue supercompetent cells (Stratagene)

Supercompetent *Escherichia coli* XL1-Blue cells (Stratagene) were thawed on ice. 100µl were transferred into a pre-cooled, sterile Falcon 2059 tube. After addition of 1.7µl 1.42M β-mercaptoethanol, the tube was incubated for 10min on ice with gently shaking every 2 minutes. 2-4µl of the ligation mix (see 2.1.6) were added and the cells incubated for 30min on ice and heat shocked for 45-50sec at 42°C in the water bath. The cells were incubated for 2min on ice and 900µl SOC, pre-warmed to 42°C, were added. Then this suspension was incubated for 60min at 37°C and 50-200µl were plated on LB-plates with 50 or 100µg/ml ampicillin, respectively. The plates were incubated over night at 37°C.

2.1.7.3 Long term storage of bacteria

From each positive clone 500µl of the bacterial overnight culture were transferred into a 2ml microcentrifuge tube and mixed with 500µl glycerol freezing solution and stored at -80°C

Glycerol freezing solution (30ml)

22.5ml Glycerol 87% (sterile)
3.75ml MilliQ
750µl 1M Tris HCl, pH 8.0
3ml 1M MgSO₄

2.1.8 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a technique to amplify a selected sequence of a DNA template using specifically designed primers and a thermostable DNA polymerase.

2.1.8.1 In vitro Mutagenesis, Gene SOEing

Gene SOEing stands for gene splicing by overlapping extensions (Horton et al. 1990; Horton 1997). This is a PCR based approach to introduce mutations or tags into a gene sequence.

Example: Mutation of the alpha1 subunit

The *Bam*HI restriction site between the transmembrane domain 3 and 4 of the alpha1 subunit gene was deleted (silent mutation). This mutation was necessary to avoid a cleavage of the alpha1 subunit gene during subsequent cloning steps.

The construct α_1 pCi was used as a template for generating two PCR-fragments containing the silent mutation (*Bam*HI restriction site deleted). PCR product 1 (primer pair alpha1-Bcl-F and alpha1-mut-R3, 1130bp) and PCR product 2 (primer pair alpha1-mut-F3 and pCi-R3, 460bp) were mixed and used as template for the third PCR performed with the primer pair alpha1-Bcl-F and pCi-R3 (1558bp). The resulting PCR product was digested with the enzymes *Bcl*I and *Eco*RI and ligated into the *Bgl*II and *Eco*RI digested modified pUC18 vector (3.3.1.2).

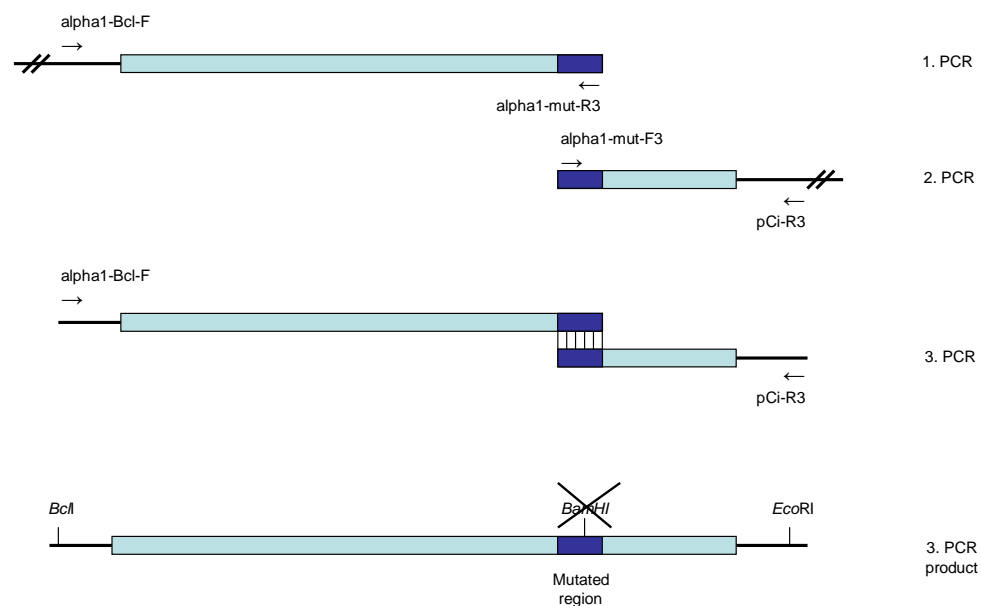


Fig. 10: Schematic drawing of Gene SOEing. Three PCR steps were necessary to delete the *Bam*HI restriction site between the transmembrane domain 3 and 4 of the alpha1 construct. This was performed by introducing a silent mutation in the overlapping primer sequences. The resulting third PCR product was digested with the enzymes *Bcl*I and *Eco*RI and ligated into the *Bgl*II and *Eco*RI digested modified pUC18 vector.

2.1.8.2 PCR screening of recombinant DNA clones

Well isolated single colonies were picked from agar plates and resuspended in 80µl ddH₂O. 40µl of this suspension were transferred into 500µl LB-medium containing

the required antibiotic and incubated at 37°C in a Thermomixer (Eppendorf). The remaining 40µl were denatured (5min at 95°C and 5min on ice) and centrifuged at 15,700 x g for 2min at room temperature. 5µl of the denatured DNA were used for screening PCR. As a positive control 0.5µl of the ligation mix were used. As a negative control 5µl ddH₂O instead of DNA were used.

PCR reaction mix (4 samples)

10µl 10x buffer QIAGEN
8µl dNTP mix (2.5mM each)
2µl Primer A (10pmol/µl)
2µl Primer B (10pmol/µl)
0.4µl Taq DNA polymerase (5U/µl QIAGEN)
57.6µl MilliQ to a total of 80µl
+5µl denatured DNA to 20µl mix

PCR conditions

1min 94°C
15sec 94°C
15sec 55°C
1min 72°C
29 cycles

The PCR product was analyzed by agarose gel electrophoresis.

Positive clones were selected for plasmid DNA preparation. For an overnight culture 20ml LB medium containing the appropriate antibiotic were inoculated with 20µl of positive clone suspension and incubated at 37°C on a shaking incubator. Plasmid DNA was purified as described in 2.1.1.1 and sequenced (VBC-Biotech, Vienna).

2.2 Insect cell culture

2.2.1 Cultivation of Sf9 insect cells

Sf9 cells were grown in serum-free InsectXpress medium (LONZA). For maintaining the cell line, Sf9 cells were cultivated in cell culture flasks (Nunclon, VWR) at 27°C (Incubator Type B 6120, Heraeus). They were seeded at 0.45×10^6 cells/ml and passaged when reaching a confluent layer (usually after 3 to 4 days). Adherently

grown cells were detached from the surface by shaking the flasks or by repeated aspirating with the pipette.

For mid scale production and infection of cells, the Sf9 cells were grown as suspension cultures in InsectXpress medium, containing an antibiotic mixture (100U/ml Penicillin, 100U/ml Streptomycin and 0.25µg/ml Amphotericin, Lonza, Walkersville, MD USA). Cells were cultivated in sterile Erlenmeyer flasks (Corning) in a shaking incubator (Multitron II, INFORS AG) at 27°C and 140rpm.

Cells were grown to a cell density of about 3.0×10^6 cells/ml and diluted to 0.45×10^6 cells/ml every 2 to 3 days. The density of the cells was determined with a "Neubauer improved" counting chamber (Marienfeld). The viability of the cells was determined by mixing the cells with 0.4% Trypan Blue-solution (GIBCO). Trypan Blue can only be accumulated by dead cells and thus dead cells appear violet and can be counted when visualized in a microscope.

For infection, cells were used at a density of $2\text{--}2.5 \times 10^6$ cells/ml and 5% fetal calf serum (FCS) were added to the InsectXpress medium to slow down degradation of the viruses by proteases. The cells were harvested two to three days post infection ($4,600 \times g$, 15min, 4°C). The cell pellets were stored at -80°C for further experiments, like Western blot analysis (2.6.1), radioligand binding assays (2.3) or receptor purification experiments (2.4, 2.5).

For long-term storage log-phase Sf9 cells (~80% confluent layer) were harvested ($2,000 \times g$, 5min, 4°C) and resuspended and diluted in freezing medium (InsectXpress + 15% DMSO, 4°C) to an end concentration of about $1\text{--}2 \times 10^7$ cells/ml. Cells were transferred to freezer vials and after slow freezing stored in liquid nitrogen. Usually, Sf9 cells were substituted by freshly thawed cells after passage 35 (after about 3 month).

Sf9 cells were thawed by placing the vial directly into a water bath at 37°C for about 2 minutes. The content of the vial was then transferred into a Nunc tissue culture flask containing 10ml of InsectXpress medium. Cells were allowed to settle for 30 minutes at room temperature and afterwards incubated at 27°C. After 4 hours the medium and floating cells were drawn off and 10ml of fresh medium were added to the flask and incubation at 27°C continued. When cells became ~90% confluent, monolayer culture was split 1:3 and after one to two weeks normal culturing (1:10) could be continued.

2.2.2 Transfection of Sf9 cells to produce recombinant viruses

To produce recombinant baculoviruses, Sf9 cells were co-transfected with transfer plasmid DNA and Bac Magic™ DNA (Novagen).

For each co-transfection 2ml Sf9 cells in BacVector Insect Cell Medium (Novagen) were seeded with a density of 0.45×10^6 cells/ml into a well of a 6-well plate. During the one hour incubation period the transfection mixture was prepared.

Transfection mixture

1ml BacVector Insect Cell Medium
5µl Gene Juice (Novagen)
5µl BacMagic DNA (100ng total)
5µl Transferplasmid (500ng total)

All components were assembled in the order listed in a sterile tube and incubated at room temperature for 15-30min to allow complex formation. Immediately after medium was removed from the cells, 1ml transfection mixture was added dropwise to the center of the well. The plate was then incubated at 27°C in a moist bag. After 24h 1ml of BacVector Insect cell medium was added.

After 5 days of incubation the supernatant was collected, centrifuged (10min, $4,100 \times g$, 4°C) and transferred into a sterile tube. 10% FCS were added and the recombinant virus stock was stored in the dark at 4°C.

2.2.3 Amplification of recombinant baculovirus

Amplification of the recombinant viruses is necessary to generate high titer viral stocks for subsequent experiments. Sf9 cells were grown to a density of 2×10^6 cells/ml and infected with a low moi (multiplicity of infection) of about 0.01-0.02. The flasks were incubated with shaking (27°C, 140rpm) until cells were well infected (4-5 days). The cells were harvested by centrifugation (10min, $2,000 \times g$, 4°C) and 10% FCS were added to the supernatant. The recombinant virus stock was stored in the dark at 4°C. The stocks were labeled as followed:

<u>Initial virus stock</u>	Recombinant baculovirus stock generated by co-transfection of Sf9 cells
<u>Intermediate stock (IS)</u>	Low moi amplification of initial virus stock
<u>Working stock (WS)</u>	Low moi amplification of IS

2.2.4 Virus PCR

By isolating the virus-DNA of a working stock and subsequent amplifying a specific part of this DNA by PCR, it was possible to determine whether the virus stock contained amplified viruses at all and also to estimate the amount of these viruses. With this method it was possible to get a first information about the success of the virus amplification but whether the amplified viruses are infectious or not had to be determined by plaque assays (2.2.5).

For Virus DNA isolation the GFX PCR DNA and Gel Purification Kit from GE Healthcare was used according to the protocol of the manufacturer. Briefly, 100µl virus solution were incubated with 600µl capture buffer for 15min at 60°C in a Thermomixer (1400rpm). The mixture was applied on a GFX column, incubated for 1min at room temperature and centrifuged (1min, 15,700 x g, room temperature). The column was washed with 300µl washing buffer and DNA was eluted with 50µl EB buffer (pre-warmed to 72°C).

For PCR, 5µl DNA were mixed with 20µl PCR-mastermix containing 1mM dNTPs, PCR buffer (QIAGEN), 0.5U Taq-polymerase (QIAGEN) and 5pmol of each primer of the appropriate Specific primer pairs for the amplification of full length α_1 , β_3 and γ_2 encoding viruses listed below.

α_1 : forward α_1 -33XhoI, reverse α_1 +252
 β_3 and β_3 His: forward β_3 -24-XbaI, reverse β_3 +242-XhoI
 γ_2 and γ_2 His: forward γ_2 -38XhoI, reverse γ_2 +188-R

<u>PCR conditions</u>	1min 94°C
	15sec 94°C
	15sec 55°C
	1min 72°C
	28 cycles

5µl of the PCR product were analyzed by agarose gel electrophoresis (2.1.4).

2.2.5 Virus plaque assay

Virus titers were determined by virus plaque assay and expressed as plaque forming units per ml (pfu/ml).

Sf9 cells from a shaking culture were diluted to 0.45×10^6 to 0.5×10^6 cells/ml and 2ml of this dilution seeded in each well of a 6-well plate. The plate was incubated for at least 1 hour at 27°C. In the meantime 10-fold dilutions of the virus stock were prepared by mixing 70µl viruses with 630µl InsectXpress-medium in sterile tubes (10^{-1} - 10^{-7}). After the cells had attached to the plate the medium was aspirated and cells from each well were incubated with 500µl of the virus (10^{-2} - 10^{-7}).

After the viruses have been allowed to infect the cells, the supernatant was aspirated and the cells were covered with 2ml of an agarose-overlay. For this agarose-overlay 3 vol. 1.3x Sf-900 medium (Novagen, pre-warmed to 37°C) were mixed with 1 vol. melted 4% agarosegel (Novagen) in a 100ml flask and incubated for a minimum of 30min at 37°C.

After the agarose overlay has solidified (20min at room temperature) the plates were incubated in a zip lock bag containing a moistened towel for 6-7 days at 27°C.

To visualize the plaques, 1ml MTT solution (1mg/ml Insect Xpress medium) was dropped on the cells and incubated for 45min in the dark in the incubator. After that time the yellow MTT stain not accumulated by the living cells was removed. The plates were incubated for 1 hour at 27°C and afterwards for 16 hours in the dark at room temperature.

Clear plaques indicating cells that died due to an infection starting from a single virus, can be distinguished from the dark background of the living cells (see Fig. 11).

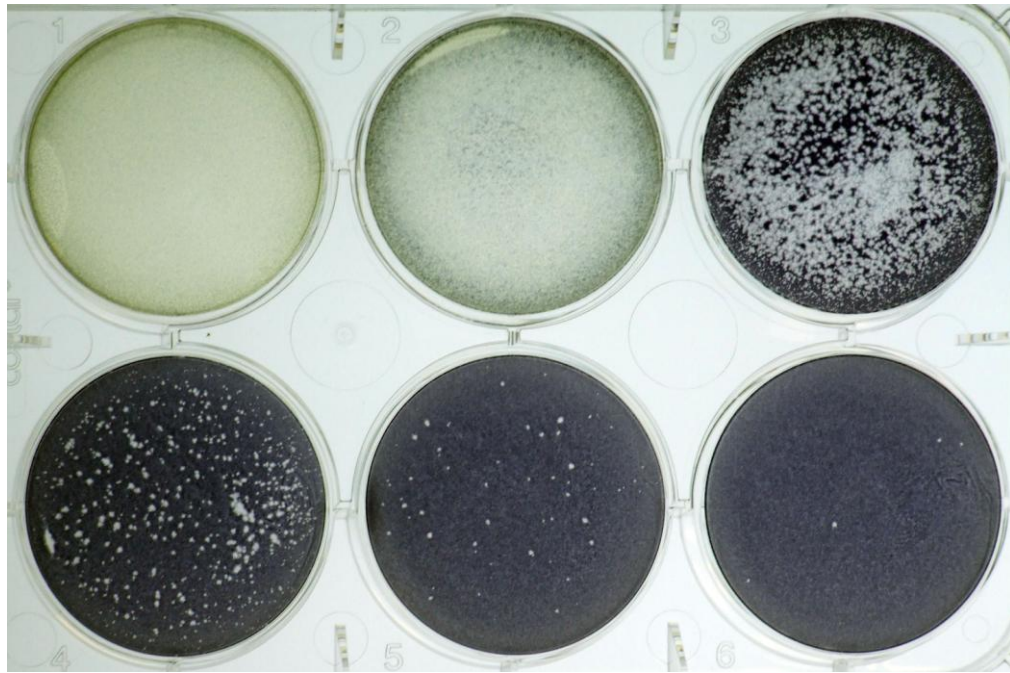


Fig. 11: Picture of a virus plaque assay. In this 6-well plate Sf9 cells were infected with decreasing amounts of baculoviruses (from the left upper part 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7}). The cells were stained with MTT after 6 days post infection. Colourless plaques were expressed as plaque forming units per ml (pfu/ml).

2.2.6 Recombinant baculoviruses encoding for GABA_A receptor subunits α_1 , β_3 and γ_2

Recombinant baculoviruses encoding for a single GABA_A receptor subunit were generated by Leila Wabnegger as described in her thesis “High yield expression and His-tag purification of recombinant GABA_A receptors using the Sf9-baculovirus Expression Vector System”, 2005.

The DNA fragments encoding the full-length α_1 and β_3 subunit were cloned into the pVL1393 transfer vector, those encoding the full-length γ_2 subunit into the pVL1392 transfer vector (Fig. 12).

All the subunit encoding fragments were cloned behind the strong virus promoter polyhedron, which is a very late phase promoter. Transfection of Sf9 cells with linearized virus-genome DNA and the generated virus transfer plasmid led to homologous recombination, resulting in recombinant viruses encoding for the GABA_A receptor subunits (Fig. 12) (Wabnegger 2005).

As a means for purification of GABA_A receptors it was decided to clone the His-tag on the N-terminal side of the β_3 and γ_2 subunit. The larger N-terminal extracellular domains are necessary for the formation of extracellular binding pockets.

In front of the eight histidine residues the first four amino acids of the mature protein were added. This was done because it is not clear whether the signal protease which cleaves the signal peptide also needs those first amino acids for recognizing its restriction site. To allow for a subsequent removal of the His-tag, a factor Xa-cleavage site (Ile-Glu-Gly-Arg) was added behind the histidine residues, separated by two amino acid residues (Gly-Thr) forming a KpnI-restriction site. The whole tag was generated by PCR and then cloned into a subunit encoding vector (Fig. 13) (Wabnegger 2005)

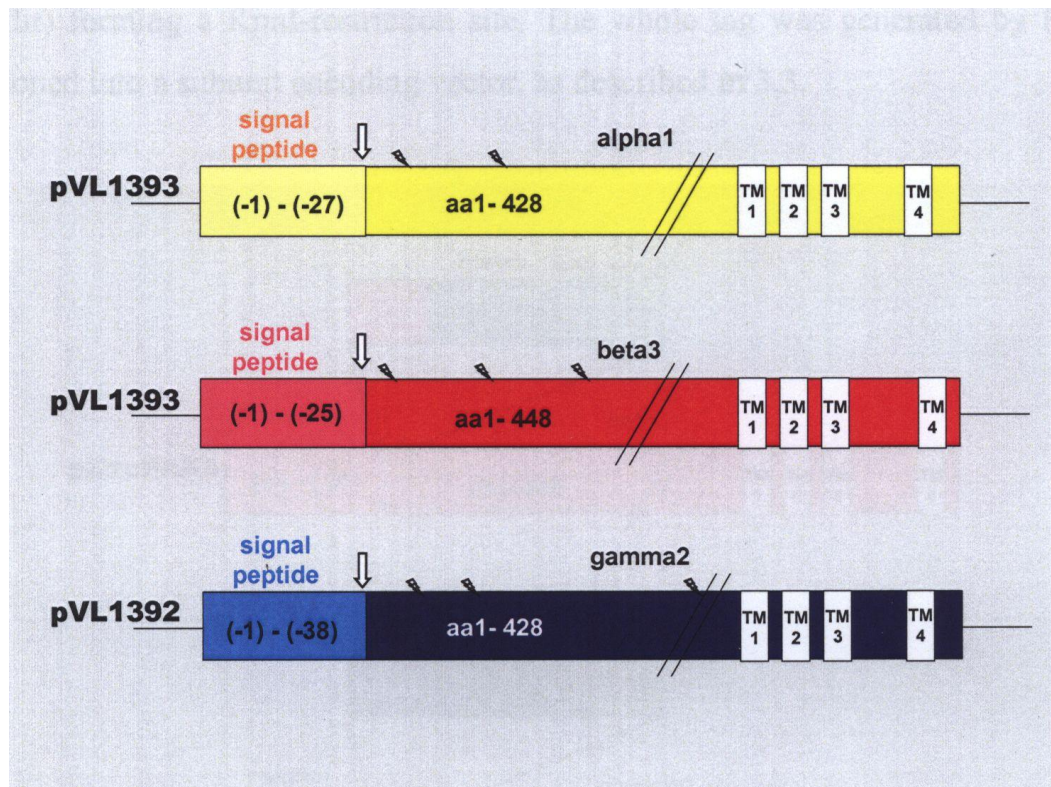


Fig. 12: Schematic drawing of recombinant baculovirus transfer vectors containing either α_1 , β_3 or γ_2 encoding sequences. The subunit encoding sequences are cloned into the following baculovirus transfer vectors: α_1 and β_3 : pVL1393, γ_2 : pVL1392. (TM: transmembrane domain, aa: amino acid). The arrow indicates where the signal protease cleaves the signal peptide away, resulting in the mature protein starting with amino acid one. The little tails indicate the glycosylation sites of the appropriate subunits (Wabnegger 2005).

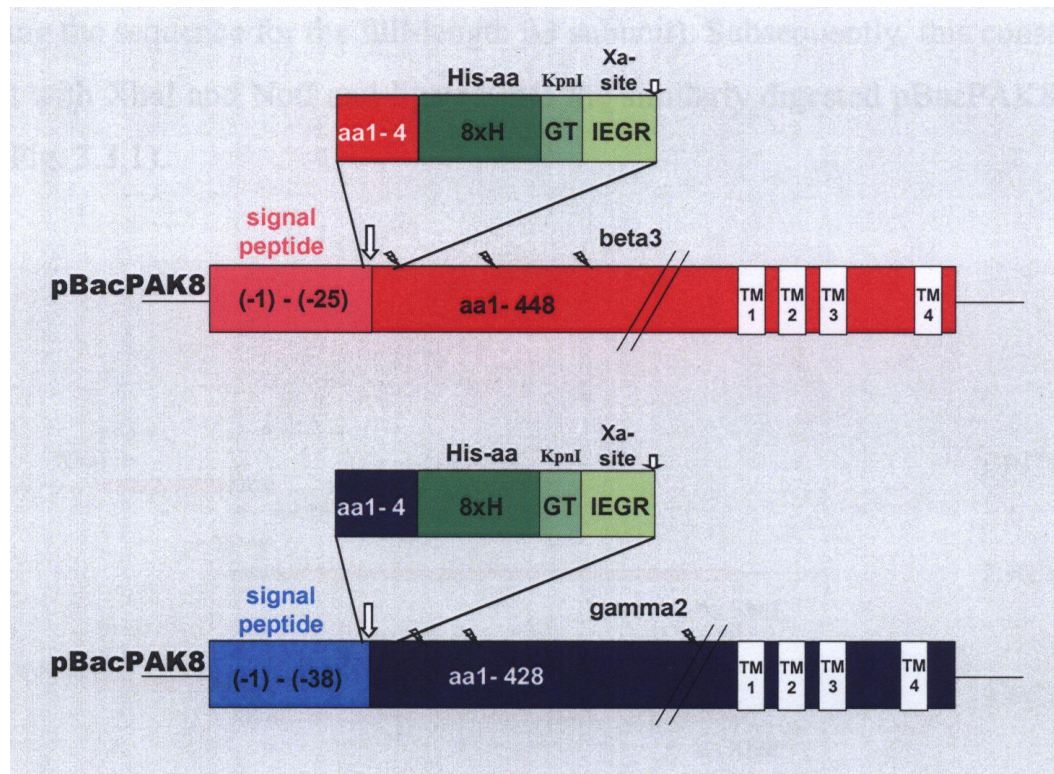


Fig. 13: Schematic drawing of recombinant baculovirus transfer vector (pBacPAK8) containing either the His-tagged β_3 or γ_2 encoding sequences. The His-tags are situated between the signal peptide and the first amino acid of the mature proteins. In the schematically drawn His-tag, amino acids are represented by single letter code (Wabnegger 2005).

2.3 Radioligand Binding Studies

Radioligand binding studies were performed to determine the binding site density (B_{\max}) of receptors (either from membrane preparations or immunoprecipitations for antibody characterization) as well as the affinity ($1/K_d$) of a radioactively labeled ligand for a receptor. In these experiments, a saturated concentration of the radioactively labeled ligand was incubated with the receptor of interest, until steady state binding conditions were reached, producing increasing concentrations of ligand-receptor complex. The ligand-receptor complex is classically referred to as “Bound”, meaning the amount of ligand bound to the receptor. The unbound ligand is referred to as “Free”, meaning the amount of ligand that is free and able to interact with the receptor (Fig. 14).

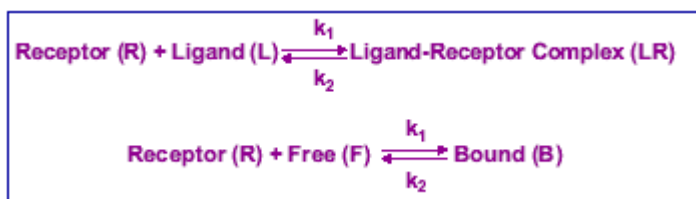


Fig. 14: Schematic representation of the basic principles of receptor binding studies

(www.tocris.com).

Unfortunately, radioactive ligands usually bind not only to the site that is being studied and is therefore referred as the specific binding, but also to non-specific binding sites. Non-specific binding sites may be constituents of the tissue or sites on glass fiber filters and usually are sites exhibiting low affinity for the ligand. In order to distinguish specific binding from non-specific binding sites, a second set of incubations was run simultaneously using the radioactive ligand and an unlabeled ligand at a concentration ($100\text{-}1000 \times K_d$) sufficient to block the binding of the radioactive ligand to the specific, but not to the unspecific sites. The amount of binding in the absence of unlabeled ligand is referred to as total binding. Binding in the presence of unlabeled ligand is referred to as unspecific binding or blank. The difference between these two is the specific binding.

The equations for analysis of a saturation experiment are based on the assumption that the free concentration of the radioligand does not change, therefore less than 10% of the radioligand should be bound to the tissue. The equation for the resulting hyperbola is:

$$\text{Bound} = \frac{B_{\max} \times \text{Free}}{K_d + \text{Free}}$$

Fig. 15: Equation to calculate bound ligand to the receptor (www.tocris.com).

K_d is the concentration where 50% of the receptors are occupied by radioactive ligand. K_d is also a measure of the affinity of a ligand (drug) for a receptor, and is equal to k_2/k_1 , where k_1 is the association rate and k_2 is the dissociation rate constant. As the concentration of radioligand increases, a point is reached where the amount of ligand bound no longer increases. This is the B_{\max} value and is a measure of the density [pM] or the amount [fmol/mg total protein] of the receptor in that tissue preparation (Fig. 16).

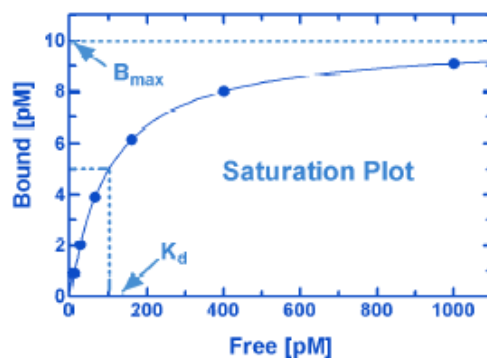


Fig. 16: Illustration of a saturation blot (www.tocris.com).

Data was analyzed using the computer program Graph Pad Prism® from Graph Pad Software, Inc. Unspecific binding was determined at every second concentration of radioactive ligand used and then interpolated by linear regression. Specific receptor binding data were then further analyzed by non-linear regression.

2.3.1 Preparation of Sf9 membranes for radioligand binding studies or SDS-PAGE

Pellets from 10ml Sf9 cells were homogenized in 10ml 50mM Tris/Citrate buffer (TC50), with an Ultraturrax. This suspension was centrifuged for 20min at 150,000 x g, 4°C. The supernatant was discarded and the membrane pellet homogenized in 5ml TC50 with the Ultraturrax.

Aliquots for subsequent radioligand binding studies (2.3) were used immediately, the protein concentration was measured by BCA protein assay (Pierce) and the aliquots for SDS-PAGE (2.6) were centrifuged 30min at 13,000 x g 4°C and the pellets stored at -20°C.

2.3.2 [³H]Muscimol binding assay

Tritium [³H] labeled Muscimol is a compound that binds in the already published GABA pocket of GABA_A receptors (Sieghart 1995; Johnston 1996; Barnard et al. 1998). This pocket is between an alpha and a beta subunit in the extracellular domain of the receptor. With this ligand alpha and beta-containing GABA_A receptors can be determined.

For this binding assay Sf9 cell membranes (see 2.3.1) were used. 300-500µl cell membrane suspension were incubated with 40nM [³H]muscimol, in the absence or presence of 1mM GABA in a final volume of 1ml. To reach the steady state the

suspensions were incubated in the dark for 60min at 4°C. Immediately afterwards the suspensions were rapidly filtered with vacuum through Whatman GF/B filters. This step is important to separate ligand-receptor complexes from free ligands. The filters were washed twice with 3.5ml 50mM Tris/citrate buffer, pH 7.1, transferred into counting vials and subjected to liquid scintillation counting (Filter-CountTM, Packard).

2.3.3 [³H]Flunitrazepam binding assay

Tritium [³H] labeled Flunitrazepam is a compound, belonging to the class of benzodiazepines, that binds in the already published benzodiazepine pocket of GABA_A receptors (Sieghart 1995; Johnston 1996; Barnard et al. 1998). This pocket is between an alpha and a gamma subunit in the extracellular domain of the receptor. With this ligand alpha and gamma-containing GABA_A receptors can be determined.

For this binding assay Sf9 cell membranes were used (2.3.1). 100µl cell membrane suspension were incubated with 10nM [³H]flunitrazepam, in the absence or presence of 100µM diazepam in a final volume of 0.5ml. To reach the steady state the suspensions were incubated in the dark for 90min at 4°C. For the following protocol see 2.3.2.

2.3.4 Radioligand binding assay with solubilized receptors

To perform a radioligand binding assay with Triton X-100 solubilized proteins, these proteins have to be precipitated before filtration. Therefore a polyethyleneglycol (PEG) precipitation assay was done.

For a [³H]flunitrazepam PEG assay 100µl sample were incubated with 50mM Tris/citrate buffer pH 7.1, 150mM NaCl, 50µg serum-albumine, 15% PEG and 10nM [³H]flunitrazepam in the presence and absence of 100µM diazepam and incubated for 90min at 4°C in the dark. The mixture was rapidly filtered with vacuum through Whatman GF/B filters, the filter washed three times with 3.5ml 8% PEG in TC50, transferred into counting vials and subjected to liquid scintillation counting (Filter-CountTM, Packard).

For a [³H]muscimol PEG assay 100µl sample were incubated with 50mM Tris/citrate buffer pH 7.1, 150mM NaCl, 50µg serum-albumine, 15% PEG and 40nM [³H]muscimol in the presence and absence of 1mM GABA and incubated for 60min at 4°C in the dark. The mixture was rapidly filtered with vacuum through Whatman GF/B

filters, the filter washed twice with 3.5ml 8% PEG in TC50, transferred into counting vials and subjected to liquid scintillation counting (Filter-Count™, Packard).

2.4 Purification of His-tagged receptors

2.4.1 Preparation of Sf9 cell membranes

Pellets of 200ml Sf9 cells were homogenized in 15ml homogenization buffer with an Ultraturrax and ultracentrifuged (150,000 x g, 20min, 4°C). The supernatant was discarded and the pellet was resuspended in 15ml washing buffer causing an osmotic disruption of vesicular structures.

Homogenization buffer

10mM Na-Phosphate
300mM Sucrose
pH 7.5
1 Complete Protease Inhibitor Cocktail
Tablet per 50ml, 4°C

Washing buffer

10mM Na-Phosphate
pH 7.5
1 Complete Protease Inhibitor Cocktail
Tablet per 50ml, 4°C

After centrifugation (48,000 x g, 4°C, 60min), the resulting pellet was resuspended in the same volume washing buffer.

An aliquot was taken for the determination of the protein concentration by BCA protein assay (Pierce). The suspension was again centrifuged for 60min at 48,000 x g, 4°C. The dry pellet was stored at -80°C.

2.4.2 Preparation of Sf9 cell membrane extracts

Frozen Sf9 cell membranes were thawed on ice and resuspended in extraction buffer (40ml/200ml Sf9 membrane pellet).

Extraction buffer

0.5% Triton X-100
1M NaCl
50mM NaH₂PO₄ pH 7.5
10-20mM Imidazole
1 Complete Protease Inhibitor Cocktail
Tablet without EDTA per 50ml, 4°C

After addition of DNaseI (Sigma, 5mg/40ml extraction buffer), the suspension was incubated under stirring for 2 hours at 4°C and centrifuged (150,000 x g, 60min, 4°C). The clear supernatant was then used for Ni-NTA affinity chromatography.

2.4.3 Purification of GABA_A receptors via a Ni-NTA column

2.4.3.1 Ni-NTA spin columns

In order to optimize the stringency of the washing conditions, small amounts of His-tagged receptors were purified by using Nickel-Nitrilotriacetic acid (Ni-NTA) spin columns (QIAGEN). The column was equilibrated with 600µl of the extraction buffer (2.4.2) and centrifuged for 2min at 700 x g at 4°C. Then the extract (1-3ml) was applied onto the column with a maximum of 600µl per centrifugation step and centrifuged for at least 5min at 270 x g at 4°C. The flow-through was collected. The column was washed with buffers containing increasing concentrations of imidazole (40mM, 80mM) and the proteins eluted twice with 200µl elution buffer. Flow-through, wash fractions and eluates were analyzed by SDS-PAGE and Western blot (2.6).

40mM imidazole buffer

40mM Imidazole pH 7.5
0.5% Triton X-100
300mM NaCl
50mM Sodiumphosphate pH 7.5

Elution buffer

250mM Imidazole pH 7.5
0.5% Triton X-100
300mM NaCl
50mM Sodiumphosphate pH 7.5

2.4.3.2 Ni-NTA column purification

Larger amounts of His-tagged GABA_A receptors were purified on Ni-NTA-agarose beads (QIAGEN) packed into empty columns (Filtration Column, Separtis). The column was packed at room temperature, washed with MilliQ water and transferred to the cold room. There the column was equilibrated with 2 bed volumes of extraction buffer (see 2.4.2). The extract was applied onto the column (6-10ml/h) and the flow-through was collected. The column was washed 2 times with 3 bed volumes extraction buffer, followed by 3 bed volumes of buffer with optimized imidazole concentration (see 2.4.3.1). The His-tagged GABA_A receptors were eluted 6 times with 1 bed volume of elution buffer containing 250mM imidazole. The eluates were collected separately and stored at 4°C for further analysis.

2.5 Benzodiazepine column-affinity-chromatography

Frozen Sf9 cell membrane preparations (see 2.4.1) were thawed and resuspended in extraction buffer. The suspension was stirred for two hours at 4°C and centrifuged (145,000 x g, 4°C, 60min). The clear supernatant (extract) was then used for affinity chromatography.

Extraction buffer

10mM HEPES, pH 7.4
300mM KCl
2% TritonX-100
1mM EDTA
1mM Benzamidine
100mg/l Bacitracin
0.3mM PMSF

Prior to use benzodiazepine column (Fuchs and Sieghart 1989) was washed with a buffer containing 10mM HEPES pH 7.4 and 0.2% Triton X-100. Then the column was equilibrated for about 6 hours with the extraction buffer. The extract was applied to the column at a rate of 7-8ml/h. The column was washed with the extraction buffer for one day with a rate of ~5ml/h, followed by 20ml of elution buffer.

<u>Elution buffer</u>	10mM HEPES
	150mM KCl
	0.5% Triton X-100
	1mM EDTA
	1mM Benzamidine
	0.5mM DTT
	100mg/l Bacitracin

Proteins bound to the column were eluted at a rate of 5ml/h with a freshly prepared elution buffer, containing the benzodiazepine tranxilium (Dipotassiumclorazepat). Therefore 4mg/ml tranxilium were dissolved in the elution buffer and the pH was adjusted to 7.6 with HEPES powder. The eluted proteins were collected in 10ml aliquots and used for subsequent experiments.

The column was washed with elution buffer without tranxilium and afterwards over night with a buffer containing 10mM HEPES and 0.2% TritonX-100, pH 7.7, followed by washing over 3-4 days with the same buffer adjusted to pH 7.4. Finally, the column was washed with 100ml of a 6M urea-solution, followed by a last washing step with 10mM HEPES and 0.2% TritonX-100 and stored at 4°C.

2.6 SDS-PAGE, Western blot analysis & Coomassie blue staining

2.6.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Invitrogen's NuPAGE manual. In a discontinuous system, proteins were separated on 10% SDS-polyacrylamide gels, consisting of a 5% stacking and a subjacent 10% separating gel.

Immediately after the separating gel was prepared, it was overlaid 2-3mm with 0.1% SDS. After 4h of polymerization at room temperature the gels could be stored at 4°C. Before using them, the gel surface was washed with 1x stacking gel buffer and overlaid with the stacking gel. Immediately, a comb was stacked into the gel avoiding air bubbles.

<u>Separating gel</u>	10% of a 30% Acrylamide/Bis solution- 29:1-(3.3% C) (BioRad) 0.1% SDS 25% 4x Separating gel buffer (1.5M Bis- Tris, pH 6.4) 0.1% Ammoniumpersulfate (APS) 0.001% Tetramethyl-ethylenediamine (TEMED)
<u>Stacking gel</u>	5% of a 30% Acrylamide/Bis solution 29:1-(3.3% C) (BioRad) 0.1% SDS 25% 4x Stacking gel buffer (0.5M Bis- Tris, pH 6.4) 0.1% APS 0.001% TEMED

After polymerization the comb was removed carefully and the slots washed with 1x NuPAGE MOPS SDS running buffer.

<u>1x MOPS SDS running buffer</u>	50mM MOPS 50mM Tris 0.1% SDS 1mM EDTA pH 7.7
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After the gel boxes were assembled and the gels were mounted, the outer chamber was filled with 600ml 1x NuPAGE MOPS SDS running buffer and the inner chamber was filled with 200ml of the same running buffer containing 500µl NuPAGE antioxidant.

Prepared membrane samples can be used directly for SDS-PAGE. Extracted proteins have to be precipitated before applying on a gel. This precipitation was performed with a chloroform/methanol precipitation. Therefore the sample was vortexed with 1 volume of methanol and ½ volume of chloroform and centrifuged for 5min at 18,000 x g, 4°C. After the centrifugation the proteins stay in the interphase. The

upper phase was removed and 2 volumes of methanol added, vortexed and again centrifuged for 30min at 18,000 x g, 4°C. The supernatant was removed and the pellet dried completely. This pellet can then be used immediately or stored at -20°C, like a membrane protein pellet, for further analysis.

The samples of interest were mixed with 1x NuPAGE LDS sample buffer and 1x NuPAGE reducing agent, denatured while shaking for 10min at 70°C in a Thermomixer, and centrifuged at 17,900 x g for 2min at room temperature. 10-20µl (1µg/µl) were loaded carefully into each slot of the stacking gel. MagicMark™ XP Western Standards (Invitrogen) was loaded as marker for the protein size into one slot. The proteins were separated with 200V (max. 125mA/gel) for 45min. When the gel run was finished, the gels could be used for Western blot analysis (2.6.2) or Coomassie blue staining (2.6.3).

2.6.2 Western blot

In the meantime PVDF membranes (polyvinylidene fluoride, Millipore Immobilon-P Transfer membrane) in the size of 6 x 8cm were moistened for 20sec in methanol, shortly washed in MilliQ ultra pure water, and stored in 1x transfer buffer containing 10% methanol until use for blotting.

10x transfer buffer

0.5M Tris
0.4M Glycine
0.02M SDS

The above mentioned gels and two Whatman filter papers (6 x 8cm) per gel were also moistened in 1x transfer buffer. The blot was assembled bubble free. The plus pole was in contact with a Whatman filter. On top of the filter there was the PVDF membrane, then the gel, again a Whatman filter and the minus pole. The proteins were transferred onto the membrane in 1x transfer buffer with 58mA/gel (max. 20V) for 1h. Thereafter the blot was disassembled and the membranes were shaken in blocking buffer for 1h at room temperature.

Blocking buffer

1.5% Dry milk powder
0.1% Tween 20
1x PBS

10x PBS

1400mM NaCl
25mM KCl
20mM KH₂PO₄
100mM Na₂HPO₄
pH 7.5

After this the remaining binding sites on the membrane should have been blocked. The blots were transferred into new boxes which contained the primary antibody of interest in a concentration of 1µg/µl (diluted in blocking buffer). They were kept overnight at 4°C shaking.

On the next day the blots were washed three times with blocking buffer for 20min and incubated with the secondary antibody (alkaline phosphatase conjugated AffiniPure F(ab')₂ Fragment goat anti-rabbit IgG (H+L), Jackson Immuno Research Laboratories) diluted 1:2,000 in blocking buffer for 1h at room temperature. To remove the secondary antibody another three washing steps in blocking buffer for 20min each were performed. After that another two final washing steps with assay buffer were done (5min and 10min).

Assay buffer

0.1M Diethanolamine
1mM MgCl₂
pH 10.0

Finally the blots were overlaid with the substrate CDP-Star (Applied Biosystems, Bedford, MA) and diluted 1:1,000 in assay buffer. After the blots were exposed in the Fluor-S Multi-Imager (Bio-Rad Laboratories, Hercules, CA), the reaction of alkaline phosphatase with CDP-Star could be visualized and the emerging chemiluminescent signals could be quantified by densitometry. The signal intensity was evaluated with Quantity One®.

To establish the linear range of the detection system, antibody generated signals were measured at a range of protein and antibody concentrations. Immunoreactivities were within the linear range under the used experimental conditions, thus permitting a direct comparison of the amount of antigen between each sample of a lane. After evaluation, the blots were stored in assay buffer at 4°C.

2.6.3 Coomassie blue staining

After an SDS-PAGE (2.6.1) the proteins can be stained unspecifically in the polyacrylamide gel. Therefore the gel was washed three times with MilliQ water. About 20ml staining solution (Simply Blue™ Safestain, Invitrogen) per gel were used and incubated for 1 hour. Then the staining solution was discarded and the gel washed with MilliQ water for several hours or over night, until the background is clear or light blue. The gel with the stained protein bands can be scanned for further quantitative measurements.

2.7 Generation and purification of polyclonal antibodies

For all methods to be used antibodies were needed, which specifically detect different subunits of the GABA_A receptor. Some of these antibodies were directed against small peptides, others against fusion proteins representing sequences that are unique for the respective subunit. Immunizations with antigens were done in rabbits.

2.7.1 Peptide antibodies

The peptide for the subunit $\alpha 1(1-9)$ was custom synthesized containing an additional N- or C-terminal cysteine (piCHEM, Graz, Austria), which was coupled to either keyhole limpet hemocyanin or to Thiopropyl Sepharose 6B (Amersham Pharmacia Biotech). The keyhole limpet hemocyanin (KLH) enhances immunogenicity in immunized rabbits. The coupling to the Thiopropyl Sepharose 6B is necessary to obtain purified antibodies.

2.7.2 Fusion protein antibodies

The GABA_A receptor subunit sequences of interest were $\alpha 1(328-382)$, $\beta 3(1-13)$, $\beta 3(345-408)$, $\gamma 2(1-33)$ and $\gamma 2(319-366)$. These sequences had already been cloned into the pMALc vector (New England Biolabs, Beverly, MA) and by that were fused with the maltose binding protein (MBP), an enhancer of immunogenicity and were additionally tagged with seven histidines to enable purification with Ni-NTA agarose (Qiagen). The fusion proteins resulting from expression of the vector product in bacteria were used for immunization of rabbits. The same sequences had also been cloned into a modified vector pGEX-4T3 (Amersham Pharmacia Biotech), which in addition to glutathione S-transferase (GST) encodes for seven C-terminal histidines.

Fusion proteins obtained by expression of this vector were coupled to Affigel 10 (BioRad) and were used for purification of antisera and separation from antibodies directed against MBP.

2.7.3 Expression of fusion proteins

The above mentioned vectors encoding for fusion proteins were transformed into E.coli XL1 blue (Stratagene, La Jolla, CA). For expression E.coli bacteria were cultured overnight (Thermomixer 180-200rpm at 37°C) in 20ml LB-rich medium, containing 50µg/ml ampicillin. 4ml of the overnight culture were transferred into 250ml of fresh pre-warmed (37°C) LB-rich medium containing 50µg/ml ampicillin and grown (Thermomixer 250rpm at 37°C) to an OD₆₀₀ of 0.5-0.7. The expression of the fusion protein was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG, end concentration should be 1mM) and the bacteria were again incubated and grown to an OD₆₀₀ of 1.6. The cells were incubated for 10min on ice and harvested by centrifugation (15,000g 15min 4°C). The pellets were kept at room temperature for 30min and stored overnight at -20°C. After thawing, the pellets were extracted in 1ml per 50ml culture with 6M guanidine-HCl in 1x QE during intensive stirring for 1h at room temperature.

5x QE

0.5M Na₂HPO₄ x 2 H₂O
3M NaCl
pH 8.0

The suspension was then centrifuged (43,000 x g 30min at 20°C) and the supernatant was applied to a Ni-NTA agarose column. The column was washed five times with 2.5ml 8M urea in 1x QE at pH 6.3. The fusion protein was eluted by washing five times with 0.5ml 8M urea in 1x QE at pH 5.9 and six times with 1ml 8M urea in 1x QE at pH 4.5. Finally the purity of the eluates was controlled on a 10% polyacrylamide gel (2.6.1) and the eluates were stored at 4°C.

For immunization, MBP fusion proteins were diluted in 1x PBS. GST fusion proteins, used for coupling to Affigel 10, were brought to pH 7.0 - 7.5.

2.7.4 Immunization of rabbits

In order to obtain specific antibodies directed against defined subunits of the GABA_A receptor, several rabbits were immunized either with MBP fusion proteins or KLH coupled peptides. For this, 100µg of the fusion protein or KLH coupled peptides

were used and diluted in 1x PBS to a total volume of 750µl. In practice 1.5ml of this solution, sufficient for two animals, were emulsified with 2ml of complete Freund's adjuvant (for the first immunization), containing 1mg/ml inactivated and dried mycobacterium tuberculosis (Sigma) that functions as a booster for the immune reaction, or with 2ml of incomplete Freund's adjuvant (for all following immunizations) and injected into the rabbits.

Every three to five weeks the immunization was repeated until the titer or the specificity of the antibodies decreased. Ten to fourteen days after each immunization cycle the rabbits were bled and then every week until the next immunization. The antibodies were characterized by Western blot, immunoprecipitation, and/or immunohistochemistry analysis.

2.7.5 Purification of antibodies

To obtain clear blood serum the blood cells and platelets were pelleted by centrifugation (4,600 x g 2h at 4°C). The supernatant was stored overnight at 4°C and centrifuged (48,000 x g 30min at 20°C) again the next day. In the meantime the required GST columns were brought to room temperature and washed with 20ml 1x PBS. After centrifugation, the supernatant was diluted with 1/9 volume of 10x PBS, applied to an appropriate immunoaffinity column, and chromatographed. The efflux was collected and stored at 4°C for a possible second chromatography. To remove unbound protein, the column was washed with 50ml 1x PBS. The bound antibodies were eluted in ten to fifteen 1ml fractions with alkaline elution buffer.

<u>Alkaline elution buffer</u>	100mM Na ₂ HPO ₄
	150mM NaCl
	pH 11.5

Each fraction was directly eluted into 100µl of acidic neutralization buffer and mixed rapidly to immediately neutralize the pH.

<u>Acidic neutralization buffer</u>	1M NaH ₂ PO ₄
	pH 4.4

The protein concentrations of the purified antibodies were measured with a bicinchoninic acid (BCA) protein assay kit (Pierce) according to manufacturer's

instruction. After freezing the antibodies with liquid nitrogen they were stored at -20°C. The used column was washed several times: 20ml elution buffer, 20ml 1x PBS, 20ml elution buffer with the opposite pH that was used for the elution and again with 30-50ml 1x PBS. The column was stored in 1x PBS containing sodium azide at 4°C.

3 RESULTS

This thesis is based on the results of the thesis from Leila Wabenegger, in which the possibility of expressing $\alpha_1\beta_3\gamma_2$ -containing recombinant GABA_A receptors in Sf9 cells was evaluated. With the present experiments we wanted to establish large scale production of pure, non degraded $\alpha_1\beta_3\gamma_2$ GABA_A receptors for further structural analysis, such as crystallization, photoaffinity labeling and surface plasmon resonance investigations.

3.1 $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors

In the first experiments already cloned and tested single viruses for the α_1 , β_3 or γ_2 subunits were used. A His-tag had been cloned into the β_3 subunit by Leila Wabenegger for an additional option to purify the receptor with a Ni-NTA column.

3.1.1 Determination of the optimal conditions for the expression of $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors

Previous experiments had shown that the ratio of the three viruses used for co-infection had to be in a certain range for the production of correctly assembled receptors. To establish this range Sf9 cells were co-infected with different combinations of viruses, harvested three days post infection (dpi) and subjected to [³H]flunitrazepam binding assays. Then specific binding was calculated (Tab. 3).

In addition, different multiplicities of infection (moi) were used. The multiplicity of infection defines the ratio between viruses and Sf9 cells. For instance, a moi of 1 means that, each Sf9 cell on average was infected with a single virus particle, in the case of a moi of 3 each cell was infected with three virus particles, etc. This factor could also influence the receptor expression level, because huge amounts of viruses could lead to degraded receptors or to premature cell death.

Another parameter in recombinant protein expression with Sf9 insect cells was time. It was necessary to find out the optimal time point for harvesting the cells after expression of the recombinant receptor, when the expression level was high but unspecific degradation of the expressed recombinant protein was still low. Normally the protein expression was stopped after 72 hours (3 days) (Fig. 17).

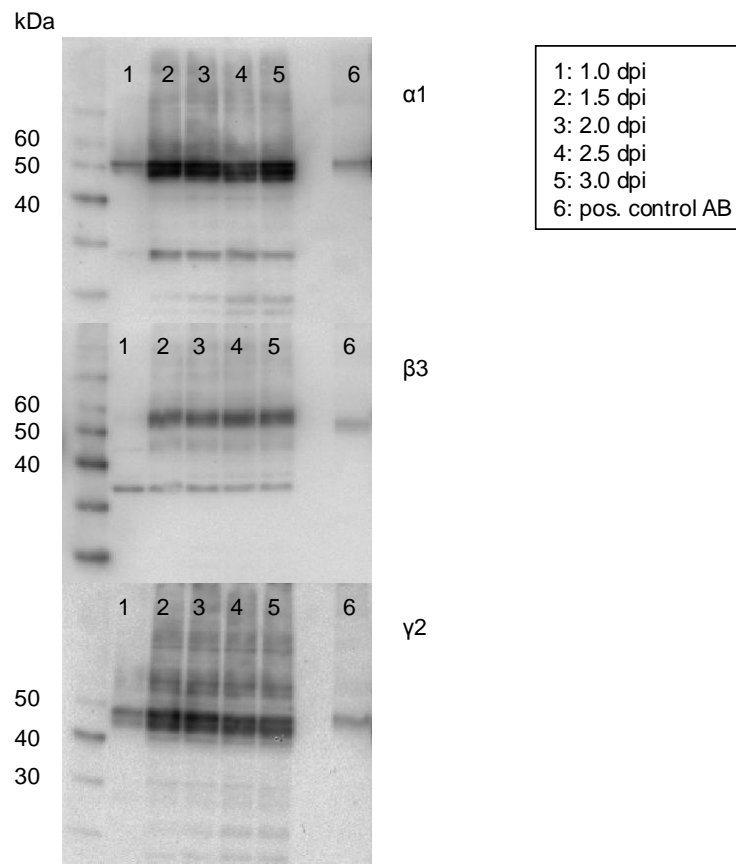


Fig. 17: Western blot experiment of different harvesting time points of an $\alpha_1\beta_3\text{His}8\gamma_2$ expression in Sf9 cells. Sf9 cells were co-infected with α_1 , $\beta_3\text{His}8$ and γ_2 subunits and harvested after 1.0, 1.5, 2.0, 2.5 and 3.0 days post infection (dpi). Membranes were prepared and the same amount of total protein was subjected to an SDS-PAGE and Western blot analysis with subunit specific α_{1L} , β_{3N} and γ_{2L} antibodies performed.

The α_1 subunit was already expressed after 24h (1.0 dpi, lane 1) at the apparent molecular mass of 50kDa compared to the positive control of the antibody in lane 6. After 1.5, 2.0, 2.5 and 3.0 days post infection the expression level seemed to be high and the amount of expressed protein stable. Additionally, degraded α_1 bands were stained at about 45kDa in lane 2-5, which were overlapping with the α_1 band. The other protein bands at 20 and 30kDa were cross-reactivities of the antibody.

The β_3 subunit was not stained after 24h (lane 1). This subunit was expressed quite well from 1.5-3.0 days post infection (lane 2-5) and stained at the apparent molecular mass of 55kDa, compared to lane 6, the positive control of the antibody. Due to the His-tag, the expressed $\beta_3\text{His}8$ subunit runs at a higher molecular mass in the SDS-PAGE compared to the positive control. Again a degradation product of the subunit was slightly stained in lane 2-5 at the apparent molecular mass of 45kDa. The stained bands at 35kDa were cross-reactivities of the used antibody.

The γ_2 subunit could be stained and therefore was expressed from 24h on in all samples at the apparent molecular mass of 48kDa. The correct molecular mass of the γ_2 subunit could be compared to the positive control of the antibody in lane 6. Similar to the other two subunits, the γ_2 subunit showed degradation at about 45kDa. The γ_2 antibody unspecifically stained protein bands from 15-40kDa. The cross-reactivity-bands of the α_1 L, β_3 N and γ_2 L specific antibodies used in this thesis were already checked and determined before. Bands at higher molecular masses indicated aggregates of the subunits, which were not dissolved properly during SDS-PAGE.

This experiment indicated that the α_1 , the β_3 and the γ_2 subunit were expressed at a high level after 36h (1.5dpi). The intensity of the specific bands between 1.5 and 3.0 days post infection seemed to be the same, meaning that the expression of the protein could not be enhanced by more time.

The degradation could not be diminished by harvesting the cells at an early time point (24h). The GABA_A receptor subunits were expressed under a late promoter in the Sf9 cells and therefore needed a certain time span to be expressed and the receptor to be assembled. To confirm these Western blot results, a [³H]flunitrazepam binding assay was performed (Tab. 2).

dpi	pmol [³ H]flunitrazepam/mg total protein
1.0	0.2
1.5	0.9
2.0	1.0
2.5	1.2
3.0	1.0

Tab. 2: [³H]flunitrazepam binding assay experiment at various harvesting time points of Sf9 cells, expressing $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors. Sf9 cells were co-infected with α_1 , $\beta_3\text{His}8$ and γ_2 subunits and harvested at different days post infection (dpi). Membranes were prepared, receptors were incubated with 10nM [³H]flunitrazepam \pm 100 μ M diazepam and specific binding (pmol [³H]flunitrazepam/mg total protein) was calculated.

The binding assay provided similar results as the Western blot (Fig. 17). At a harvesting time point of 24h after infection (1.0 dpi), the expression level was very low. After 36h (1.5 dpi) the expression reached 1.0 picomol receptor per mg total protein and remained constant with a small increase to 1.2pmol at 2.5 dpi.

To reach a high expression level and to keep the degradation of the proteins low, we decided to harvest the Sf9 cells between 2-3 days post infection, but this had to be tested for different constructs or ratios of subunits.

Knowing the appropriate harvesting time point of the Sf9 cells, the next expression experiments were performed to investigate different ratios of subunits for a high receptor expression level (Tab. 3). Membranes from harvested Sf9 cells were prepared (2.3.1) and the amount of receptors in a sample was determined with radioligand binding assays with 10nM [3 H]flunitrazepam (2.3.3), in the presence and absence of diazepam. The resulting counts per minute (cpm) were converted to pmol receptor binding sites per mg total protein (Tab. 3).

Ratio α_1 : β_3 His8 : γ_2	moi	pmol [3 H]flunitrazepam/mg total protein
2:1:1	1	3.1
2:1:1	2	3.2
3:1:1	1	2.6
3:1:1	2	2.7

Tab. 3: Optimization of the protein expression of $\alpha_1\beta_3$ His8 γ_2 GABA_A receptors in Sf9 cells. Sf9 cells were co-infected with different ratios of α_1 , β_3 His8 and γ_2 subunits and different moi (multiplicity of infection). Cells were harvested after 3 days and membranes were prepared. Receptors were incubated with 10nM [3 H]flunitrazepam \pm 100 μ M diazepam and specific binding (pmol [3 H]flunitrazepam/mg total protein) was calculated.

The resulting pmol [3 H]flunitrazepam/mg total protein indicated the amount of GABA_A receptors containing α_1 , β_3 and γ_2 subunits. The used ratios (2:1:1 and 3:1:1) and moi (1 and 2) showed no significant difference in the receptor expression level. Under all conditions approximately 3pmol $\alpha_1\beta_3$ His8 γ_2 GABA_A receptors were expressed. The variation of the expressed 3pmol and the expressed 1pmol shown in Tab. 2, lie in the same expression level range.

We decided to use both ratios 2:1:1 and 3:1:1 with a moi = 1 and a harvesting time point of 72 hours (3 days).

3.1.2 One-step purification with a Ni-NTA column

Proteins, containing a Histidine (His)-tag consisting of a minimum of six His, can be purified over a Nickel-Nitrilotriacetic acid (Ni-NTA) column. Bound proteins can then be eluted from the column by competitive displacement with increasing amounts of imidazole.

For our experiments a pellet of 330ml harvested Sf9 cells, expressing $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors, was used. This pellet contained 240pmol [³H]flunitrazepam binding sites and 2200pmol [³H]muscimol binding sites. With the assumption, that 1pmol receptor is equal to 250ng receptor, 240pmol were equal to 60µg receptor protein (Tab. 4).

Membranes were prepared from the Sf9 pellet (2.4.1), extracted in 80ml 0.5% Triton X-100 extraction buffer (2.4.2) and the clear supernatant was applied to a 750µl Ni-NTA agarose column. The column was washed with 4ml of a 40mM imidazole buffer and then with 1ml aliquots of an 80mM, 100mM, 120mM and 150mM imidazole buffer. The proteins were eluted with 5 x 1ml 200mM imidazole buffer aliquots (eluates 1-5).

The yield of the washing and elution steps was determined with PEG precipitation and 10nM [³H]flunitrazepam or [³H]muscimol radioligand binding assays. The purity of the receptor was tested with an SDS-PAGE with subsequent Coomassie blue staining (Fig. 18). The radioligand binding assay data are summarized in Tab. 4.

The binding assay results indicated a disequilibrium of $\alpha_1\beta_3\gamma_2$ ([³H]flunitrazepam) to $\alpha_1\beta_3$ ([³H]muscimol) containing receptors in the isolated membranes. Only about 10% of the GABA receptors contained a γ subunit as indicated by the ratio of pmol [³H]flunitrazepam compared to pmol [³H]muscimol binding.

The one-step purification also indicated a massive loss of receptors. The membrane extract contained 235pmol [³H]flunitrazepam binding sites or 60µg receptor protein; 186pmol of these receptors were retained by the column and after elution only 77pmol or 20µg receptor protein remained. This indicated either a loss or a partial inactivation of 2/3 of the receptors. Thus only 1/3 of the receptors was purified in a way able to bind [³H]flunitrazepam and was then concentrated in 5ml.

	pmol [³ H]flunitrazepam	pmol [³ H]muscimol
Membrane preparation	240	2200
Extract	235	-
Retention	186	-
All eluates	77	1054
Eluate 1	8	112
Eluate 2	26	232
Eluate 3	32	353
Eluate 4	8	213
Eluate 5	3	144

Tab. 4: One-step purification of $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors with a Ni-NTA column. Sf9 cells were co-infected with α_1 , $\beta_3\text{His}8$ and γ_2 subunits and harvested after 3 days. Membranes and extracts were prepared and receptors purified via a Ni-NTA column. Samples from different stages of the purification were incubated with 10nM [³H]flunitrazepam \pm 100 μ M diazepam or 40nM [³H]muscimol \pm 1mM GABA and specific binding (pmol [³H]flunitrazepam or pmol [³H]muscimol) was calculated.

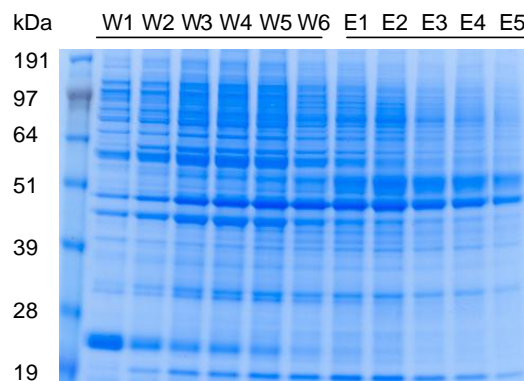


Fig. 18: SDS-PAGE and Coomassie blue staining of the one-step purification of $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors with a Ni-NTA column. Sf9 cells were co-infected with α_1 , $\beta_3\text{His}8$ and γ_2 subunits and harvested after 3 days. Membranes and extracts were prepared and receptors purified via a Ni-NTA column. Aliquots from different washing (W1-W2: 40mM, W3: 80mM, W4: 120mM, W5-W6: 150mM imidazole) and elution steps (E1-E5: 200mM imidazole) were precipitated and subjected to SDS-PAGE and Coomassie blue staining.

The Coomassie blue staining of the one-step purification of $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors in Fig. 18 showed an increasing staining of a protein band at the apparent molecular mass of 55kDa in the elution steps E1-E5, representing the $\beta_3\text{His}8$ subunit.

The signal of the α_1 subunit at about 50kDa in the eluates was partially covered by that of another protein of the same size in all samples. The γ_2 subunit at the apparent molecular mass of 48kDa was not stained with Coomassie blue, indicating that purified receptors contained mainly α_1 and β_3 His8 subunits. These subunits can form functional β_3 His8 homo-oligomeric and $\alpha_1\beta_3$ His8 hetero-oligomeric GABA_A receptors. The correct molecular mass of the subunits was also confirmed by Western blot analysis, where α_1 , β_3 and γ_2 specific antibodies were used (not shown). All other stained bands on the Coomassie blue gel presented other, non GABA_A receptor proteins from the Sf9 extract, which also were retained by and eluted from the column.

Both, the radioligand binding assay (Tab. 4) and the Coomassie blue staining results (Fig. 18) of the purification of $\alpha_1\beta_3$ His8 γ_2 GABA_A receptors with a Ni-NTA column, showed that more β_3 His8 homo-oligomeric and $\alpha_1\beta_3$ His8 hetero-oligomeric receptors than $\alpha_1\beta_3$ His8 γ_2 GABA_A receptors were expressed and purified via the His-tag with a Ni-NTA column.

3.1.3 Two-step purification with a benzodiazepine-affinity column and a subsequent Ni-NTA column

To avoid enrichment of β_3 His8 and $\alpha_1\beta_3$ His8 receptors and to enhance the purification of $\alpha_1\beta_3$ His8 γ_2 receptors, a two-step purification with a benzodiazepine and a subsequent Ni-NTA column with more material was performed. Benzodiazepines bind to the α/γ interface, so only $\alpha_1\beta_3\gamma_2$ receptors were retained by this column. The receptors could be concentrated and further purified afterwards with the Ni-NTA column.

3.1.3.1 Benzodiazepine-affinity column

For the purification with a benzodiazepine column, a pellet of 6360ml harvested Sf9 cells, expressing $\alpha_1\beta_3$ His8 γ_2 GABA_A receptors was used. This pellet contained 2820pmol [³H]flunitrazepam binding sites corresponding to 700 μ g receptor protein. Membranes were prepared from the Sf9 pellet (2.4.1) and extracted in 580ml 0.5% Triton X-100 extraction buffer (2.4.2). The clear supernatant was applied to a benzodiazepine-affinity column (2.5). The yield of the washing and elution samples was determined by PEG precipitation and [³H]flunitrazepam radioligand binding assay (2.3.3). The radioligand binding assay data are summarized in Tab. 5.

The benzodiazepine-affinity column retained 2085pmol [³H]flunitrazepam binding sites from 2380pmol of the binding sites in the extract (Tab. 5). After the elution

340pmol receptors or 85µg receptor protein remained. Thus, about 15% of the receptors ($[^3\text{H}]\text{flunitrazepam}$) from the extract were purified.

	pmol $[^3\text{H}]\text{flunitrazepam}$	Calculated µg receptor protein
Membrane preparation	2820	700
Extract	2380	595
Flow through & washing	295	-
Retention	2085	
All eluates	340	85

Tab. 5: Benzodiazepine-affinity column purification of $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors. Sf9 cells were co-infected with α_1 , $\beta_3\text{His}8$ and γ_2 subunits and harvested after 3 days. Membranes and extracts were prepared and receptors purified via a benzodiazepine-affinity column. Samples from different stages of the purification were incubated with 10nM $[^3\text{H}]\text{flunitrazepam}$ \pm 100µM diazepam and specific binding (pmol $[^3\text{H}]\text{flunitrazepam}$) was calculated.

For further purification via a Ni-NTA column, the eluates were dialyzed with a 0.2% Triton X-100 buffer over night (0.2% Triton X-100, 300mM NaCl, 25mM Na-phosphate, 0.06% Asolectin, 1 Complete Protease Inhibitor Cocktail Tablet without EDTA per 50ml, 4°C).

3.1.3.2 Ni-NTA column

The 340pmol dialyzed $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors were applied to a Ni-NTA column (0). The column was washed with a 40mM and an 80mM imidazole buffer and the proteins were eluted with a 120mM imidazole buffer (eluates 1-3).

The yield and the purity of the washing and elution steps were determined with PEG precipitation and 10nM $[^3\text{H}]\text{flunitrazepam}$ radioligand binding assay. In addition an SDS-PAGE with subsequent Coomassie blue staining was performed. The radioligand binding assay data are summarized in Tab. 6.

From the Ni-NTA column about 80pmol or 25% of the applied receptor protein were eluted (Tab. 6). Thus, from 2820pmol receptor protein in Sf9 membranes, corresponding to 700µg receptor protein, 80pmol or 20µg receptor protein remained after the two-step purification. The remaining 20µg receptor protein represented 3% of the starting material and had a purity of estimated 90%.

	pmol [^3H]flunitrazepam	Calculated μg receptor protein
Applied protein	340	85
Eluates	80	20

Tab. 6: Second step of the purification of $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors using a Ni-NTA column. Purified receptors from a benzodiazepine-affinity column were dialyzed and subjected to a Ni-NTA column. The applied sample to the Ni-NTA column and the eluates were incubated with 10nM [^3H]flunitrazepam \pm 100 μM diazepam and specific binding (pmol [^3H]flunitrazepam) and receptor protein [μg] were calculated.

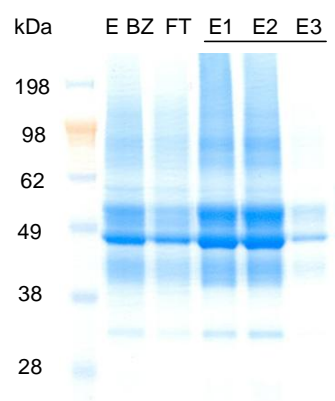


Fig. 19: SDS-PAGE and Coomassie blue staining of the purification of $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors with a benzodiazepine-affinity and a Ni-NTA column. Sf9 cells were co-infected with α_1 , $\beta_3\text{His}8$ and γ_2 subunits and harvested after 3 days. Membranes and extracts were prepared and receptors purified via a benzodiazepine-affinity column. Eluates were dialysed and loaded on a Ni-NTA column. Aliquots were precipitated, subjected to an SDS-PAGE and Coomassie blue staining. E BZ: Eluates of the benzodiazepine column, FT: Flow through Ni-NTA column and elution steps of the Ni-NTA column E1: 40mM imidazole, E2: 80mM imidazole and E3: 120mM imidazole.

The Coomassie blue staining (Fig. 19) showed highly purified $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors. There was hardly any contamination in the Coomassie blue staining. The α_1 subunit was stained at an apparent molecular mass of 50kDa, the β_3 subunit at 55kDa and the γ_2 subunit at 48kDa. Compared to the one-step purification via a Ni-NTA column, the γ_2 band was stained with Coomassie blue. The receptor was enriched comparing the benzodiazepine eluate (E BZ) to the subsequent Ni-NTA column eluates (E1-E3). The correct molecular mass of the subunits was confirmed with Western blot analysis, where α_{1L} , β_{3N} and γ_{2L} specific antibodies were used (not shown).

The purification of $\alpha_1\beta_3\text{His}8\gamma_2$ GABA_A receptors by two columns showed an enormous loss of about 97% of the expressed protein but on the other hand an extremely pure and concentrated receptor at the end.

3.2 $\alpha_1\beta_3\gamma_2$ His8 GABA_A receptors

Using the His-8 tag at the β_3 subunit not only $\alpha_1\beta_3\gamma_2$ but also $\alpha_1\beta_3$ hetero-oligomeric and homo-oligomeric β_3 receptors were formed. Using a two-step purification resulted in a highly purified $\alpha_1\beta_3$ His8 γ_2 receptor, but the loss of receptors in this procedure was huge.

To avoid these problems, the His-tag was cloned into the γ_2 subunit. When $\alpha_1\beta_3\gamma_2$ His8 receptors are expressed in Sf9 cells, only this receptor type should be purified via a Ni-NTA column. Therefore, for the next experiments, viruses encoding the α_1 , β_3 and the His8-tagged γ_2 subunit were used. It was hoped that with a single purification step a purity of the $\alpha_1\beta_3\gamma_2$ receptors similar to that of the receptors purified with two columns could be reached.

3.2.1 Determination of the optimal conditions for the expression of $\alpha_1\beta_3\gamma_2$ His8 GABA_A receptors

Before starting large expression and purification experiments of $\alpha_1\beta_3\gamma_2$ His8 GABA_A receptors via a Ni-NTA column, optimal expression conditions with a high expression level and little degradation had to be determined (Tab. 7). For that, different ratios of α_1 , β_3 and γ_2 His8 viruses, different multiplicities of infection (moi) and different harvesting times (dpi) were tested.

Sf9 cells were harvested after 2 and 3dpi. Membranes from harvested Sf9 cells were suspended in a Tris/Citrate buffer (2.3.1) and radioligand binding assays with 10nM [³H]flunitrazepam or 40nM [³H]muscimol were performed (2.3.3, 2.3.2) (Tab. 7). In addition to the binding assay experiments, the samples were also tested for degradation of receptors with SDS-PAGE and Western blot experiments (2.6) (Fig. 20).

Results (Tab. 7) indicated that there were no significant changes between different ratios of α_1 to β_3 to γ_2 His8 viruses, ratios of viruses to Sf9 cells (moi) and harvesting time points (dpi). All expression levels were around 0.5pmol [³H]flunitrazepam. This [³H]flunitrazepam expression level of $\alpha_1\beta_3\gamma_2$ His8 was lower than the [³H]flunitrazepam level of $\alpha_1\beta_3$ His8 γ_2 GABA_A receptors (1-3pmol see Tab. 2 and Tab. 3). In contrast [³H]muscimol binding data indicated an expression level around 2pmol/mg protein. The higher [³H]muscimol than [³H]flunitrazepam expression level was also found during $\alpha_1\beta_3$ His8 γ_2 expression (Tab. 4). These data indicated that under

the used conditions more receptors composed of $\alpha_1\beta_3$ subunits were formed as compared to $\alpha_1\beta_3\gamma_2$ receptors.

Ratio $\alpha_1 : \beta_3 : \gamma_2$ His8	moi	dpi	pmol [3 H]flunitrazepam /mg total protein	pmol [3 H]muscimol /mg total protein
2:1:1	1	2	0.3	2.3
2:1:1	1	3	0.5	2.0
2:1:1	2	2	0.5	1.7
2:1:1	2	3	0.5	1.6
3:1:1	1	2	0.5	2.0
3:1:1	1	3	0.5	1.8
3:1:1	2	2	0.6	2.1
3:1:1	2	3	0.6	1.8

Tab. 7: Optimization of the protein expression of $\alpha_1\beta_3\gamma_2$ His8 GABA_A receptors in Sf9 cells. Sf9 cells were co-infected with different ratios of α_1 , β_3 and γ_2 His8 subunits and different moi (multiplicity of infection). Cells were harvested after 2 or 3 days and membranes were prepared. Receptors were incubated with 10nM [3 H]flunitrazepam \pm 100 μ M diazepam or 40nM [3 H]muscimol \pm 1mM GABA and specific binding (pmol [3 H]flunitrazepam/mg total protein or pmol [3 H]muscimol/mg total protein) was calculated.

Western blot signals (Fig. 20) of the specifically stained α_1 , the β_3 or the γ_2 subunit did not indicate significant differences in the amount of subunits expressed at different ratios of α_1 to β_3 to γ_2 His8 viruses, ratios of viruses to Sf9 cells (moi) and harvesting time points (dpi).

The α_1 subunit was stained at about 50kDa and showed degradation bands overlapping with the signal of the α_1 subunit and at a molecular mass of 40kDa. Bands stained at 30kDa represented a known unspecific binding of the antibody. The β_3 subunit was stained at the molecular mass of about 50kDa. Degraded subunits were at about 40kDa and an unspecific binding of the antibody was observed at 35kDa. The γ_2 His8 subunit at an approximate molecular mass of 50kDa was also degraded, as indicated by a strong band at about 40kDa. Bands at higher molecular masses indicated aggregates of the subunits, which were not dissolved properly during SDS-PAGE.

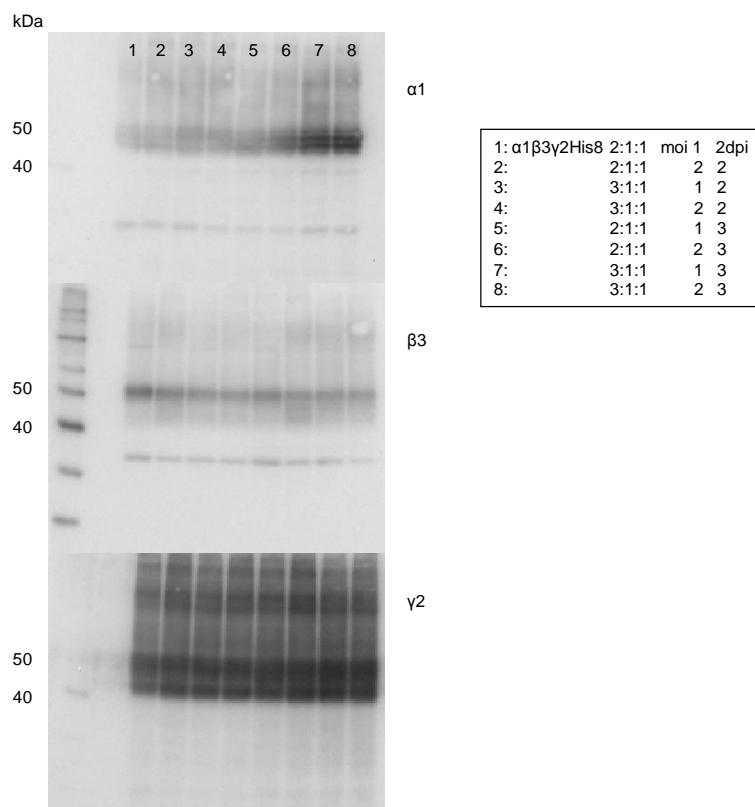


Fig. 20: SDS-PAGE and Western blot of various $\alpha_1\beta_3\gamma_2\text{His8}$ GABA_A receptor expressions. Sf9 cells were co-infected with different ratios of α_1 , β_3 and $\gamma_2\text{His8}$ subunits and different moi. Cells were harvested after 2 or 3 days, membranes were prepared and the same amount of total protein was subjected to SDS-PAGE and Western blot analysis. The blots were stained with α_{1L} , β_{3N} and γ_{2L} specific antibodies.

In spite of the strong degradation of the γ_2 subunit we decided to repeat this experiment, using a large scale purifying procedure under the assumption that the degraded γ_2 subunits might not have assembled to intact GABA_A receptors. According to the latter results, we used a ratio of 3:1:1 with a moi = 1 and harvested the cells after 2dpi = 50h to produce large amounts of $\alpha_1\beta_3\gamma_2\text{His8}$ receptors to purify them via a Ni-NTA column. The lower moi (ratio viruses to cells) and harvesting time point was chosen to keep the degradation at a minimum.

3.2.2 Purification with a Ni-NTA column

For large scale purification with a Ni-NTA column, a pellet of 1200ml harvested Sf9 cells, expressing $\alpha_1\beta_3\gamma_2\text{His8}$ GABA_A receptors was used. This pellet contained 200pmol [³H]flunitrazepam binding sites corresponding to 50 μ g receptor protein (see Tab. 8).

Membranes were prepared from the Sf9 pellet (2.4.1), extracted in 135ml 0.5% Triton X-100 extraction buffer (2.4.2) and the clear supernatant applied to a 700µl Ni-NTA agarose column. The column was washed with 3ml of a 40mM (W1-W3) imidazole buffer and then with 1ml aliquots of a 120mM imidazole buffer (W4). The proteins were eluted with 2 x 1ml 250mM imidazole buffer aliquots (E1, E2).

The yield and the purity of the washing and elution samples were determined with PEG precipitation and 10nM [³H]flunitrazepam radioligand binding assay and SDS-PAGE with subsequent Coomassie blue staining (Fig. 21) and Western blot analysis (Fig. 22). The radioligand binding assay data are summarized in Tab. 8.

	pmol [³ H]flunitrazepam binding	Calculated µg receptor protein
Membrane preparation	200	50
Extract	82	21
Flow through & washing	67	-
Retention	15	-
Eluates	2.3	0,6

Tab. 8: One-step purification of $\alpha_1\beta_3\gamma_2$ His8 GABA_A receptors with a Ni-NTA column. Sf9 cells were co-infected with α_1 , β_3 and γ_2 His8 subunits and harvested after 3 days. Membranes and extracts were prepared and receptors were purified via a Ni-NTA column. Samples from different stages of the purification were incubated with 10nM [³H]flunitrazepam ± 100µM diazepam and specific binding (pmol [³H]flunitrazepam) was calculated.

Even though the extract of a 1200ml Sf9 pellet was applied to a small column, the yield was very low (Tab. 8). From 200pmol [³H]flunitrazepam binding only 82pmol were measured in the extract, meaning a loss of 60% of receptors in this step and from these 82pmol the column retained 15pmol and 2.3pmol were eluted. These 2.3pmol corresponded with 0.6µg receptor protein.

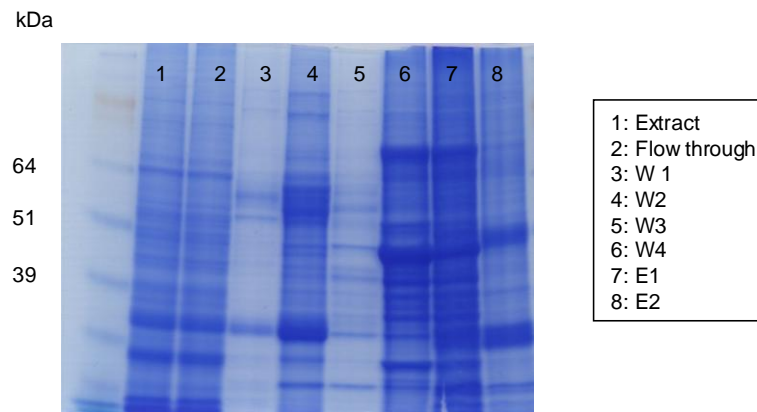


Fig. 21: SDS-PAGE and Coomassie blue staining of the $\alpha_1\beta_3\gamma_2$ His8 receptor purification with a Ni-NTA column. Sf9 cells were co-infected with α_1 , β_3 and γ_2 His8 subunits. Membranes and extracts were prepared and receptors purified via a Ni-NTA column. Aliquots from different washing (W1-W3: 40mM, W4: 120mM imidazole) and elution steps (E1-E2: 250mM imidazole) were precipitated, subjected to SDS-PAGE and Coomassie blue staining.

The Coomassie blue staining (Fig. 21) of the purification showed enriched bands in the elution steps, but due to the amount of stained bands in these lanes, it was not possible to identify the correct bands for the α_1 , the β_3 or the γ_2 His8 subunits (~50kDa). The eluted amount of receptors was too low to detect the GABA_A receptor subunits with a Coomassie blue staining. The purification was confirmed with a Western blot with subunit specific antibodies (Fig. 22).

In the Western blot of the large scale purification (Fig. 22) the α_1 , the β_3 and the γ_2 His8 subunits were stained specifically at the apparent molecular mass of 50kDa.

The α_1 subunit was found in the first washing step (W1) and in the eluate 1 (E1) and eluate 2 (E2). At about 40kDa a degradation band was stained in W4, E1 and E2. At 30kDa was an unspecific binding of the antibody. The β_3 subunit was stained specifically in the first (W1) and fourth (W4) washing and in the two elution steps (E1, E2). In these lanes degradation bands at about 45kDa were seen. The other bands at the approximate molecular mass of 35kDa were unspecific binding reactions of the used β_3 antibody. The γ_2 subunit was stained specifically in the first (W1) and last (W4) washing step and in both elution steps (E1, E2). The bands at 45, 35 and 30kDa represented unspecific binding of the used γ_2 antibody. Bands at higher molecular masses indicated aggregates of the subunits, which were not dissolved properly during SDS-PAGE.

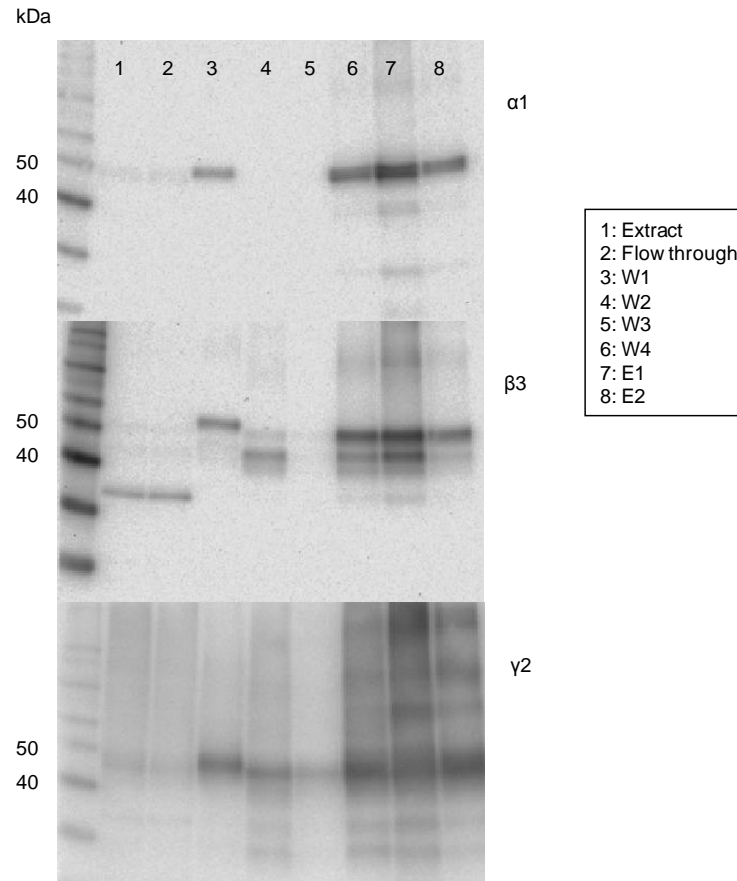


Fig. 22: SDS-PAGE and Western blot of the $\alpha_1\beta_3\gamma_2$ His8 receptor purification with a Ni-NTA column. Sf9 cells were co-infected with α_1 , β_3 and γ_2 His8 subunits. Membranes and extracts were prepared and receptors purified via a Ni-NTA column. Aliquots from different washing (W1-W3: 40mM, W4: 120mM imidazole) and elution steps (E1-E2: 250mM imidazole) were precipitated, subjected to an SDS-PAGE and Western blot analysis. The blots were stained with α_{1L} , β_{3N} and γ_{2L} specific antibodies.

These Western blot results presented a pure $\alpha_1\beta_3\gamma_2$ receptor in the first washing step, eluted with 40mM imidazole (lane 3). Unfortunately a lot of other, unspecific proteins were also eluted from the column with this low amount of imidazole (Fig. 21). The $\alpha_1\beta_3\gamma_2$ -receptor in W1 was stained with the specific antibodies, but the amount is still too low to stain with coomassie blue.

The cloning of the His-tag into the γ_2 subunit thus showed no improvement in the large scale purification via a Ni-NTA column, compared to receptors containing the His-tag in the β_3 subunit. In addition the GABA_A receptor subunits were degraded after the expression, especially the γ_2 subunit (Fig. 20). Obviously this degradation diminished the assembly and expression of $\alpha_1\beta_3\gamma_2$ -containing receptors in the Sf9 cells. The process of membrane preparation, extraction and purification with a column led to very little recovery of $\alpha_1\beta_3\gamma_2$ GABA_A receptors in the end.

Thus cloning of the His8-tag into the γ_2 subunit did not enhance the yield for the planned crystallization experiments.

The low yield of receptors might also be explained by the possibility that a single Sf9 cell was not infected simultaneously with all three subunits (α , β and γ viruses) thus reducing the formation of functional α , β and γ -containing receptors. To enhance the possibility that each cell was infected with all three subunits, the next experimental approach was to clone all three subunits into one vector, to obtain a baculovirus infecting a cell with all three subunits. In addition a His12-tag (two times 6 Histidines) was cloned into the γ_2 subunit to increase the binding of the receptor to a Ni-NTA column. This also should allow washing the column with higher concentrations of imidazole.

3.3 $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptors

The previous experiments showed several problems in the generation of high expression levels of non-degraded $\alpha_1\beta_3\gamma_2$ -containing GABA_A receptors. Receptors containing the His-tag on the β_3 subunit had an acceptable expression level but were difficult to purify and the loss of receptors during a two-step purification was huge. Due to the heterogeneity of purified receptors (β_3 His8, $\alpha_1\beta_3$ His8 and $\alpha_1\beta_3$ His8 γ_2) they could not be used for further crystallization experiments. Receptors containing the His-tag on the γ_2 subunit did not reach an expression level in the picomolar range, which would have been necessary for further structural analysis.

Another explanation for the low expression level and/or the degradation of a subunit was that both experimental setups were done with three different viruses. The chance that a cell was infected by all three viruses at the same time probably was very small. Consequently the next step was to clone all three subunits into one vector. With this method it should be ensured that an infected Sf9 cell contained all three subunit DNAs to express $\alpha_1\beta_3\gamma_2$ His12 receptors.

3.3.1 Cloning of the triple construct $\alpha_1\beta_3\gamma_2$ His12

In order to reduce the size of the plasmid for the subsequent cloning steps, the promoter region of the plasmid pBAC4x-1 was cloned into pUC18. After sequential insertion of an α_1 , a β_3 and a γ_2 subunit the completed insert was cloned back into the pBAC4x-1.

The correct DNA sequence was checked in all following cloning steps.

3.3.1.1 Cloning of the promoter region from pBAC4x-1 into pUC18

pBAC4x-1 (Fig. 23) is a baculovirus transfer plasmid designed for cloning and co-expression of up to four target genes in insect cells. The plasmid contains two polh promoters and two p10 promoters, each of which is upstream of unique cloning sites for sequential insertion of target genes. The homologous promoters are in opposite orientation to minimize recombination.

Insert: pBAC4x-1 was cut with the restriction enzymes *EcoRI* (1238) and *HindIII* (1947). The resulting DNA fragment (709bp) contained the polylinker region with two polh promoters and two p10 promoters (Fig. 23).

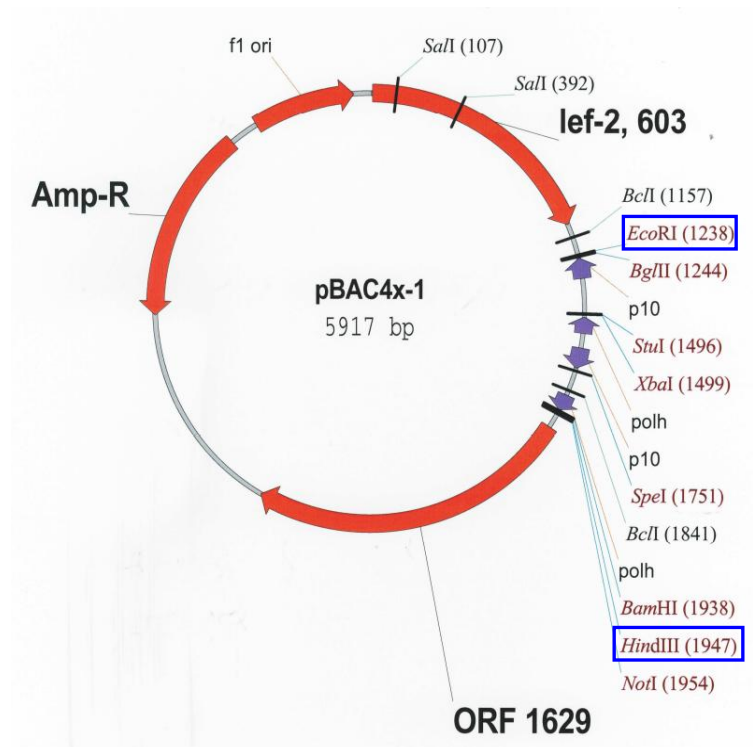


Fig. 23: Generation of the insert for cloning of the promoter region from pBAC4x-1 into pUC18.

Vector: pUC18 was cut with the restriction enzymes *HindIII* and *EcoRI* and the insert from pBAC4x-1 was cloned into the remaining vector, resulting in the construct: p10-polh-pUC18 (Fig. 24) (3345bp).

Construct: p10-polh-pUC18:

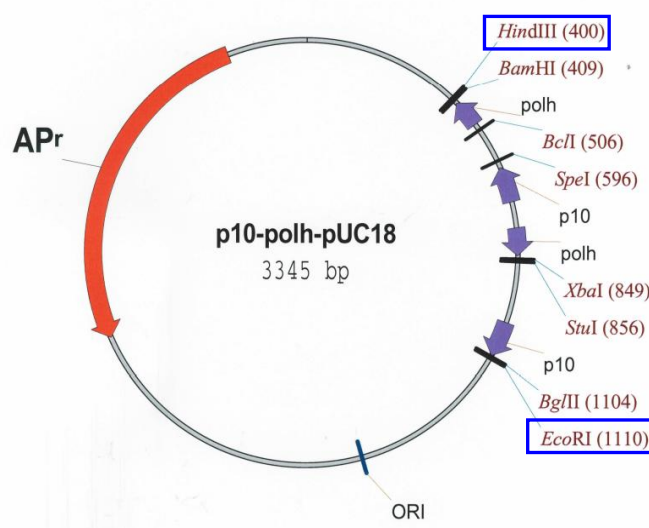


Fig. 24: Final construct after cloning of the promoter region from pBAC4x-1 into pUC18.

3.3.1.2 Mutagenesis of the alpha1 subunit and cloning of the mutated alpha1 into p10-polh-pUC18

Using Gene SOEing technique (see 2.1.8.1) a *Bcl*I site was introduced upstream of the alpha1-CDS (coding sequence) and the *Bam*HI site between TM3 and TM4 was deleted (silent mutation).

The resulting DNA fragment (insert) was cut with *Bcl*I and *Eco*RI (Fig. 25) and ligated into the vector p10-polh-pUC18, which was cut with *Bgl*II (1104) and *Eco*RI (1110) (Fig. 26), thereby generating the construct alpha1mut-p10-polh-pUC18#12 (4848bp) (Fig. 27). The restriction enzymes *Bcl*I (insert) and *Bgl*II (vector) generate compatible cohesive ends.

Insert:

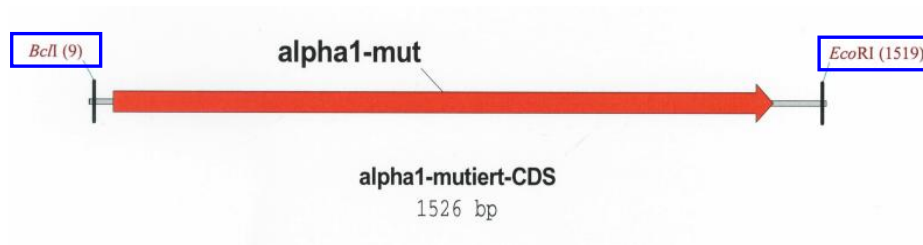


Fig. 25: Schematic drawing of the mutated alpha1 subunit used for cloning into p10-polh-pUC18.

Vector p10-polh-pUC18:

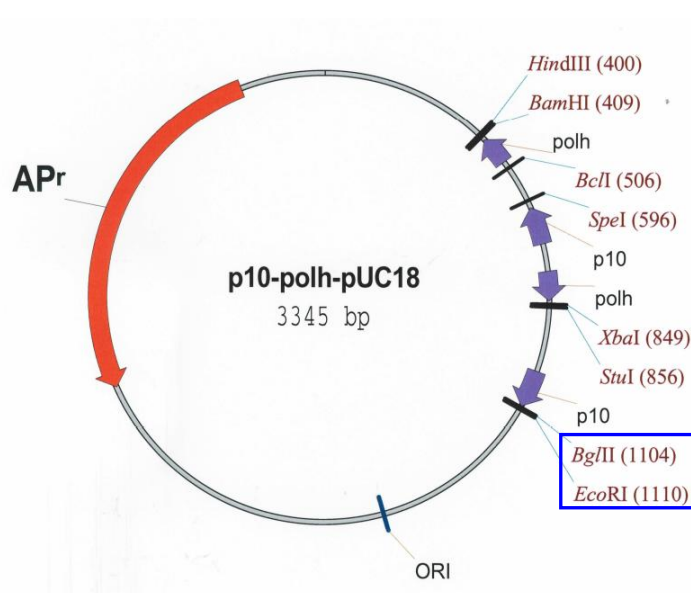


Fig. 26: Schematic drawing of the vector p10-polh-pUC18 used for cloning of the mutated alpha1 subunit.

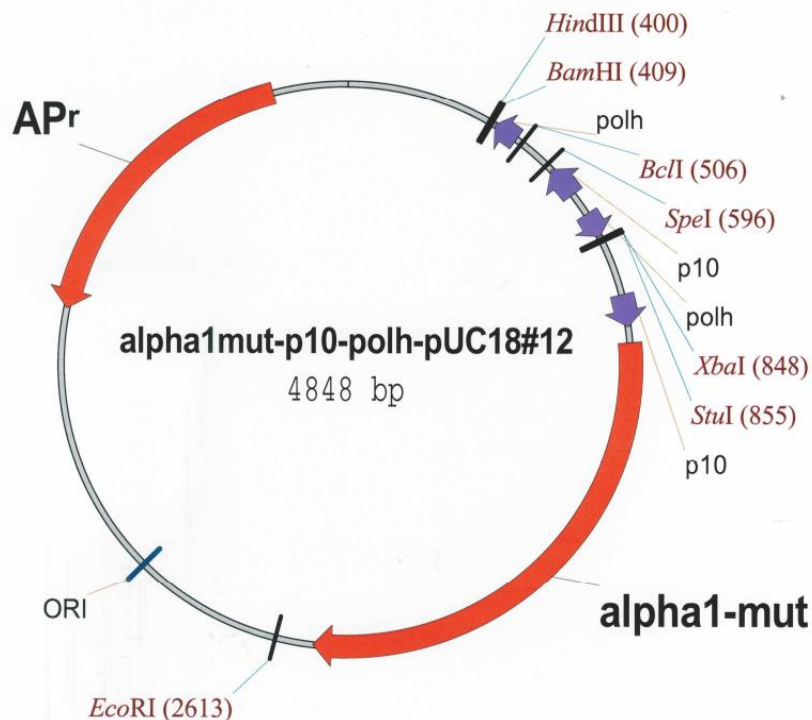
Construct alpha1mut-p10-polh-pUC18#12:

Fig. 27: Map of the final construct after cloning of the mutated alpha1 subunit into p10-polh-pUC18.

3.3.1.3 Mutagenesis of the beta3 subunit and cloning of the mutated beta3 into α_1 mut-p10-polh-pUC18

In order to prepare the beta3 subunit for the following cloning steps, four restriction sites were deleted without any change in the protein sequences of this subunit. Using Gene SOEing technique (see 2.1.8.1) the restriction sites for *Bam*HI (318) and *Eco*RI (1183) in the coding sequence of the beta3 subunit and the restriction sites for *Eco*RI (1576) and *Not*I (1569) in the 3'-untranslated region of the beta3 subunit were deleted (Fig. 28).

The resulting DNA fragment beta3-mut CDS (insert) was cut with *Xba*I and *Eco*RV and ligated into the vector alpha1mut-p10-polh-pUC18#12 cut with *Xba*I (848) and *Stu*I (855) (Fig. 29) thereby generating the construct alpha1-beta3-p10-polh-pUC18#9 (Fig. 30). The enzymes *Eco*RV (insert) and *Stu*I (vector) generate blunt ends.

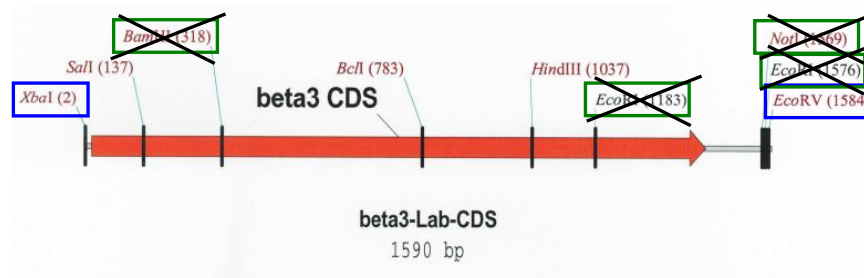
Insert:

Fig. 28: Schematic drawing of the mutagenesis of the beta3 subunit.

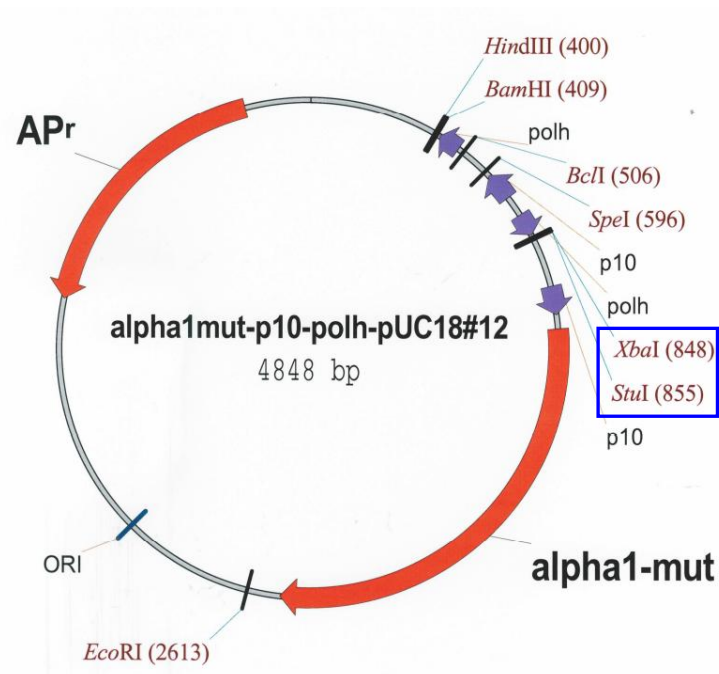
Vector alpha1mut-p10-polh-pUC18#12:

Fig. 29: Map of the vector α_1 mut-p10-polh-pUC18 used for cloning of the mutated beta3 subunit.

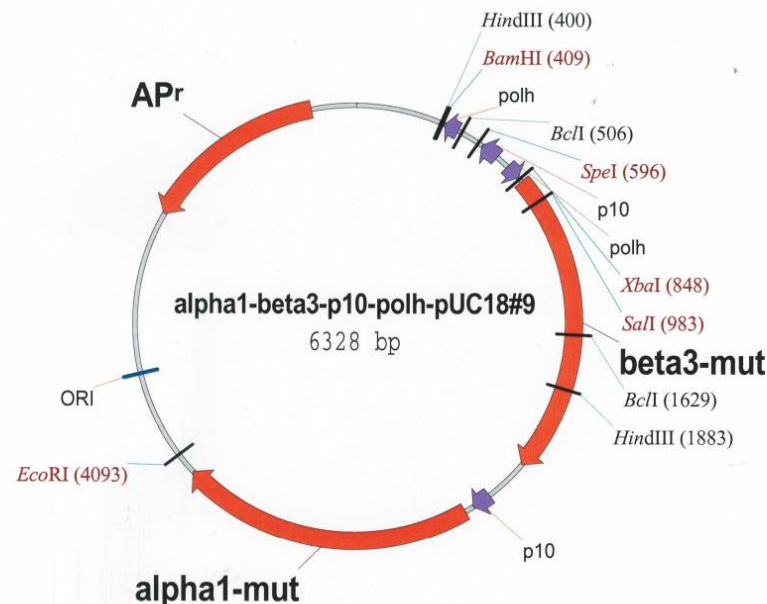
Construct alpha1-beta3-p10-polh-pUC18#9:

Fig. 30: Map of the construct after cloning of the mutated beta3 subunit into α_1 mut-p10-polh-pUC18.

3.3.1.4 Mutagenesis of the gamma2 subunit and cloning of the mutated gamma2 into α_1 - β_3 -p10-polh-pUC18

3.3.1.4.1 The mutagenesis of the gamma2 subunit was performed in three steps.

1. **Introduction of a Tobacco Etch Virus (TEV) protease recognition site.** Using Gene SOEing technique (see 2.1.8.1) the TEV protease recognition site was introduced into the gamma2-His8 CDS between amino acid 23 and 24 of the mature protein.

Resulting sequence (only the N-terminus of the mature protein is shown):

aa1-4-His8-FactorXa-aa1-23-TEV-aa24....

2. **Introduction of a double-hexa-His tag at the N-terminus.** Using Gene SOEing technique (see 2.1.8.1) a double-hexa-His tag was introduced into the gamma2-His8-TEV (step 1) construct between aa4 and 5 of the mature protein.

Resulting sequence (only the N-terminus of the mature protein is shown):

aa1-4-His6-11 amino acid spacer-His6-aa5-23-TEV-aa24....

3. **Modification of restriction sites.** To avoid a fragmentation of the gamma2 subunit by the restriction enzyme *EcoRI* in a later cloning step, the restriction site for *EcoRI* (740) in the protein coding sequence was deleted (silent mutation) by using Gene SOEing technique (see 2.1.8.1). The restriction sites for *XbaI* (1526), *SalI* (1532) and *NotI* (1543) in the 3'-UTR were deleted and replaced with a *BamHI* site (1561) (Fig. 31).

Resulting insert: gamma2-His12-TEV-mut-CDS (Fig. 32).

Insert:

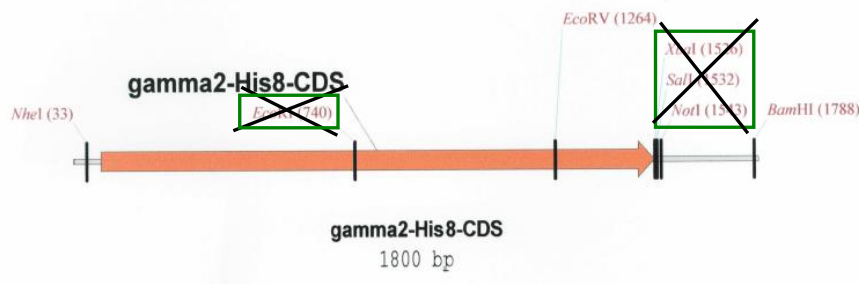


Fig. 31: Schematic drawing of the gamma2 subunit before mutagenesis.

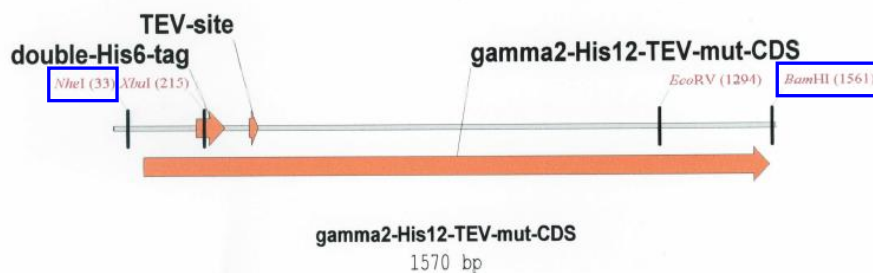


Fig. 32: Schematic drawing of the mutated gamma2 subunit used for cloning into alpha1-beta3-p10-polh-pUC18#9.

3.3.1.4.2 Cloning of the mutated gamma2 subunit into alpha1-beta3-p10-polh-pUC18#9

The plasmid alpha1-beta3-p10-polh-pUC18#9 was cut with the restriction enzymes *SpeI* (596) and *BamHI* (409) (Fig. 33). The resulting DNA fragment used as vector had a size of 6141bp. Using this combination of restriction enzymes the second polh promoter not used for further cloning steps was deleted.

The insert gamma2-His12-TEV-mut-CDS was cut with the restriction enzymes *NheI* (33) and *BamHI* (1561) (Fig. 32). The resulting DNA fragment had a size of 1528bp. The resulting construct a1-b3-g2-His12-p10-polh-pUC18 had a size of 7669bp (Fig. 34).

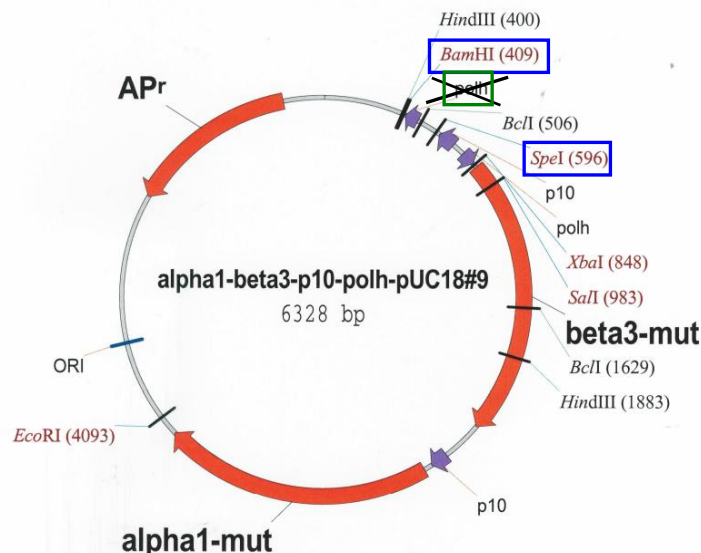


Fig. 33: Map of the vector alpha1-beta3-p10-polh-pUC18#9 used for cloning the mutated gamma2 subunit.

Construct a1-b3-g2His12-p10-polh-pUC18:

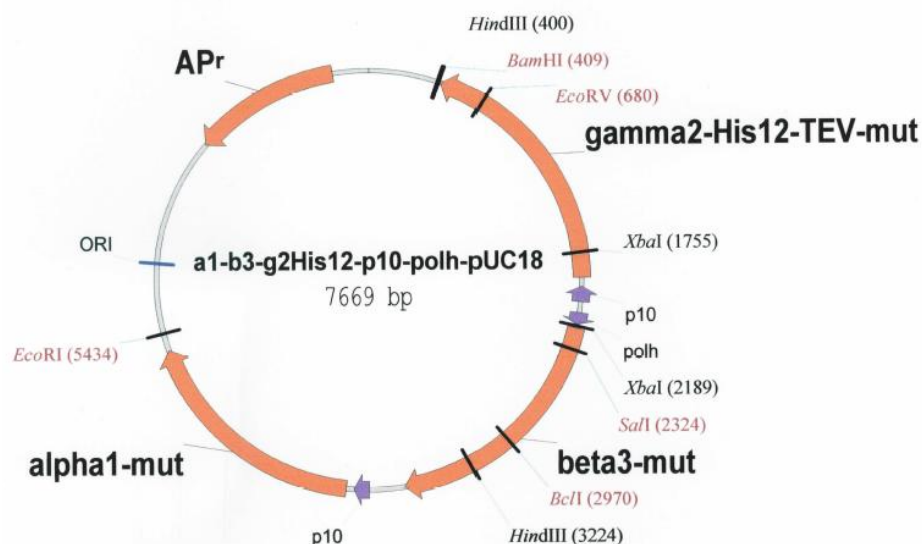


Fig. 34: Map of the final construct after cloning the mutated gamma2 subunit into alpha1-beta3-p10-polh-pUC18#9.

3.3.1.5 Subcloning of the α_1 - β_3 - γ_2 His12 construct into pBAC4x-1

The final insert encoding the three receptor subunits α_1 , β_3 and γ_2 -His12 was cut out of the pUC18 vector with the restriction enzymes *Bam*HI (409) and *Eco*RI (5434) (Fig. 35) and ligated into the similarly cut transfer vector pBAC4x-1 (Fig. 36).

The resulting plasmid construct α_1 - β_3 - γ_2 -His12-pBAC4x-1 had a size of 10242bp (Fig. 37).

Insert:

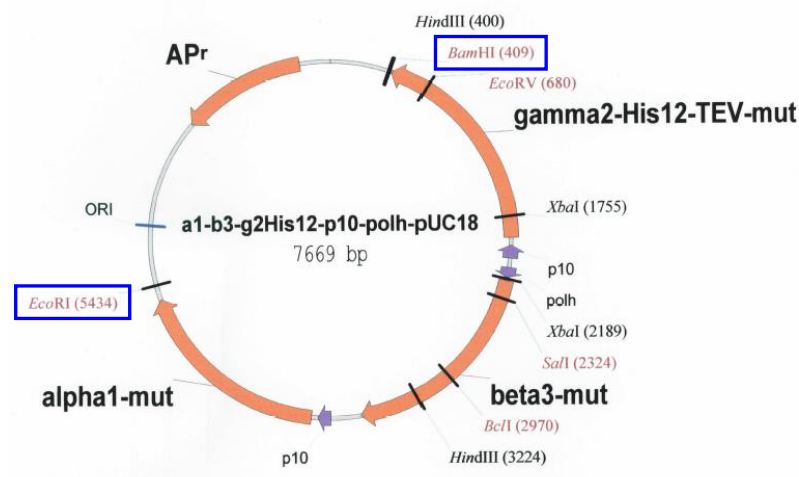


Fig. 35: Generation of the insert for subcloning of the α_1 - β_3 - γ_2 His12 construct into pBAC4x-1.

Vector pBAC4x-1:

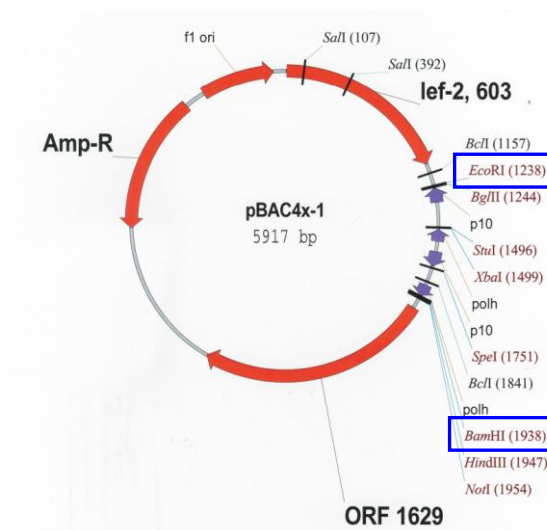


Fig. 36: Map of the vector pBAC4x-1 used for subcloning of the α_1 - β_3 - γ_2 His12 insert.

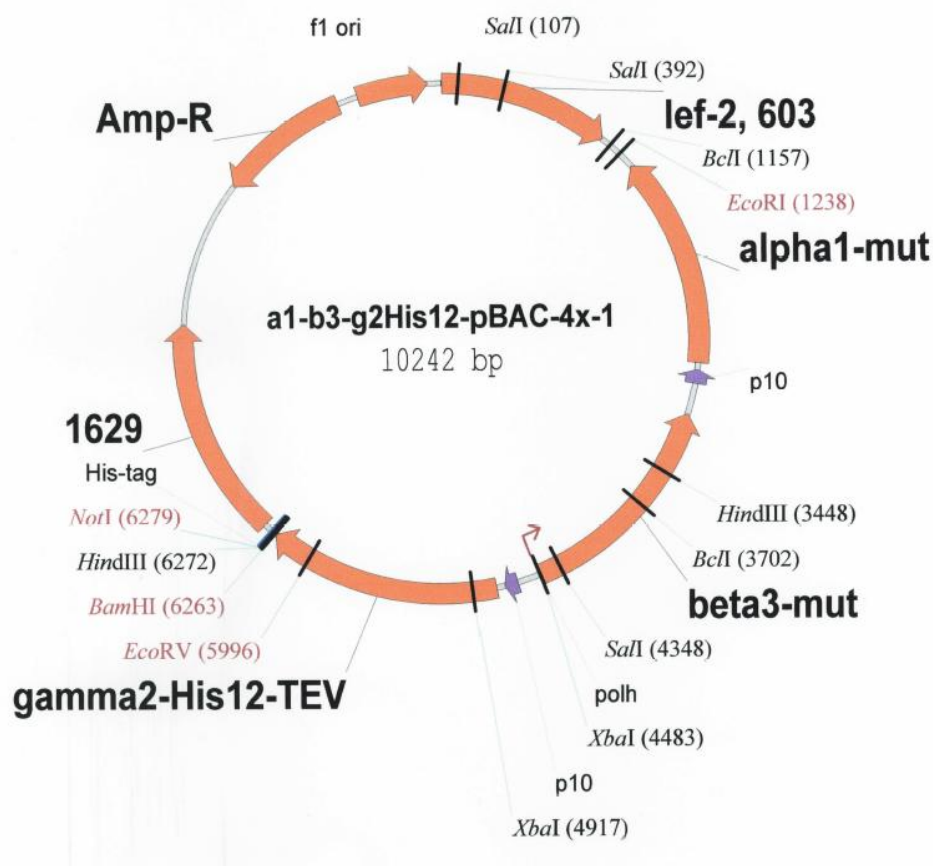
Construct α_1 - β_3 - γ_2 -His12-pBAC4x-1:

Fig. 37: Map of the final construct after subcloning of the α_1 - β_3 - γ_2 His12 DNA fragment into pBAC4x-1.

3.3.2 Determination of the optimal conditions for the expression of $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptors

Before starting large scale expression and purification experiments of $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptors via a Ni-NTA column, optimal expression conditions with a high expression level and little degradation had to be determined (Tab. 9). For that, different multiplicities of infection (moi) and different harvesting times (dpi) were tested.

Sf9 cells were harvested after 2 and 3dpi. Membranes from harvested Sf9 cells were suspended in a Tris/Citrate buffer (2.3.1) and radioligand binding assays with 10nM [³H]flunitrazepam or 40nM [³H]muscimol were performed (2.3.3, 2.3.2) (Tab. 9). In addition to the binding assay experiments, the samples were also tested for degradation of receptors with SDS-PAGE and Western blot experiments (2.6) (Fig. 38).

moi	dpi	pmol [^3H]flunitrazepam /mg total protein	pmol [^3H]muscimol /mg total protein
0.5	2	0.5	0.2
0.5	3	0.5	0.3
1	2	0.6	0.1
1	3	0.5	0.3
2	2	0.5	0.3
2	3	0.5	0.2

Tab. 9: Optimization of the protein expression of the triple construct $\alpha_1\beta_3\gamma_2\text{His12}$ in Sf9 cells. Sf9 cells were infected with the $\alpha_1\beta_3\gamma_2\text{His12}$ virus with different moi (multiplicity of infection). Cells were harvested after 2 or 3 days and membranes were prepared. Receptors were incubated with 10nM [^3H]flunitrazepam \pm 100 μM diazepam or 40nM [^3H]muscimol \pm 1mM GABA and specific binding (pmol [^3H]flunitrazepam/mg total protein or pmol [^3H]muscimol/mg total protein) was calculated.

Results (Tab. 9) indicated that there were no significant changes between different ratios of viruses to Sf9 cells (moi) and harvesting time points (dpi). All conditions showed a very low expression level 0.5pmol [^3H]flunitrazepam/mg total protein or 0.3pmol [^3H]muscimol/mg total protein. Unfortunately, these values lie below the 2pmol/mg range necessary for purification of the receptor for further structural analysis experiments.

The [^3H]flunitrazepam binding of 0.5pmol/mg total protein was comparable to the results of the single viruses expressing the $\alpha_1\beta_3\gamma_2\text{His8}$ construct (Tab. 7). In contrast, a much lower [^3H]muscimol binding expression level was obtained with the triple construct (0.3pmol) than with the $\alpha_1\beta_3\gamma_2\text{His8}$ construct (2pmol).

This low expression level of the $\alpha_1\beta_3\gamma_2\text{His12}$ receptor was also seen in the performed SDS-PAGE and Western blot, where α_{1L} , β_{3N} or γ_{2L} specific antibodies were used. The different expression conditions showed nearly the same band intensity and degradation pattern (Fig. 38).

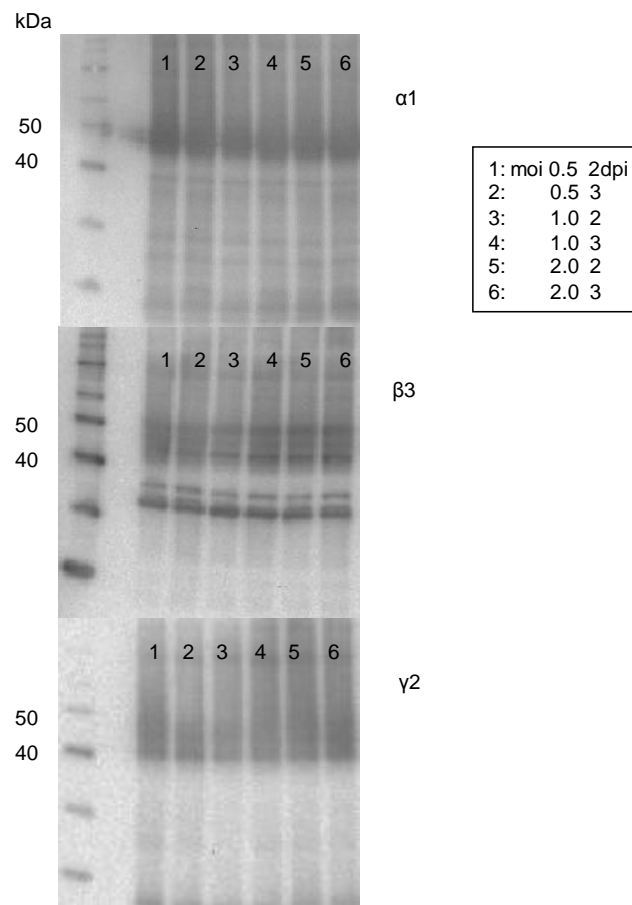


Fig. 38: SDS-PAGE and Western blot of various $\alpha_1\beta_3\gamma_2$ His12 expression conditions in Sf9 cells. Sf9 cells were infected with the $\alpha_1\beta_3\gamma_2$ His12 virus and different moi (multiplicity of infection). Cells were harvested after 2 or 3 days, membranes prepared and the same amount of total protein was subjected to SDS-PAGE and Western blot. The blots were stained with α_{1L} , β_{3N} and γ_{2L} specific antibodies.

The α_1 subunit, at an apparent molecular mass of 50kDa, was expressed quite well with a strong degradation between 45-50kDa. All other stained bands in this blot were cross-reactivity stainings of the used antibody. In contrast the β_3 subunit at about 50kDa was expressed only to a very low extent, compared to previous β_3 expression experiments. Degraded proteins of this weakly expressed subunit were stained below 50kDa by the antibody. The γ_2 subunit, visible as a strong band at the molecular mass of about 48kDa was also degraded (strong band at 40kDa). Bands at higher molecular masses indicated aggregates of the subunits, which were not dissolved properly during SDS-PAGE.

3.3.3 Purification with a Ni-NTA spin column

When the results shown in Tab. 9 and Fig. 38 were obtained, already large amounts of $\alpha_1\beta_3\gamma_2$ His12 receptors had been expressed. Although the determination of the optimal conditions showed degradation of the subunits in the membrane, we started purification experiments anyway, hoping that only non-degraded, functional $\alpha_1\beta_3\gamma_2$ His12 receptors, with a non-degraded His-tag on the γ_2 subunit would be assembled and bound to the Ni-NTA column. By using a large scale expression we hoped that the amount of lost receptors could be balanced.

To determine optimal expression conditions, a preliminary experiment with a small amount of cells for a Ni-NTA spin column purification was performed (2.4.3.1). Therefore a pellet of 170ml Sf9 cells, expressing $\alpha_1\beta_3\gamma_2$ His12 receptors was used. This pellet contained 25pmol [3 H]flunitrazepam binding sites corresponding to 6 μ g receptor protein.

Membranes were isolated from the Sf9 pellet (2.4.1), extracted in 8ml 0.5% Triton X-100 extraction buffer (2.4.2) and the clear supernatant was applied to a Ni-NTA spin agarose column (600 μ l aliquots) (2.4.3.1). The column was washed twice with 600 μ l of a 40mM imidazole buffer and then with 600 μ l of an 80mM imidazole buffer. The proteins were eluted with 2 x 200 μ l 250mM imidazole buffer aliquots (eluates 1 and 2). The samples were tested with SDS-PAGE and Coomassie blue staining (Fig. 39) and Western blot analysis (Fig. 40) for degradation.

The Coomassie blue staining (Fig. 39) showed a variety of stained bands in all washing and elution samples. There seemed to be no purification of the receptor, indicated normally by a loss of bands in the flow through or an increase of bands at the expected molecular mass in the lanes, containing the eluates. Alternatively too little receptor protein was loaded on the SDS-PAGE to be detected with Coomassie blue staining.

Therefore a Western blot with the same samples was performed, to check for a purification of at least a minimum of receptor (Fig. 40). An α_1 and a γ_2 specific antibody were used. The β_3 subunit was not investigated, because at this point we were only interested in the amount of α and γ subunits, as a measure for $\alpha_1\beta_3\gamma_2$ -subunit containing receptors.

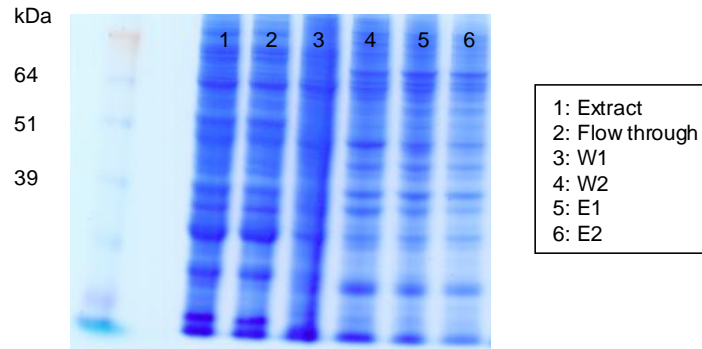


Fig. 39: SDS-PAGE and Coomassie blue staining of an $\alpha_1\beta_3\gamma_2$ His12 receptor purification with a Ni-NTA spin column. Sf9 cells were co-infected with the $\alpha_1\beta_3\gamma_2$ His12 virus. Membranes and extracts were prepared and receptors purified via a Ni-NTA spin column. Aliquots from different washing (W1-W2: 40mM imidazole) and elution steps (E1-E2: 250mM imidazole) were precipitated, subjected to an SDS-PAGE and were stained with Coomassie blue.

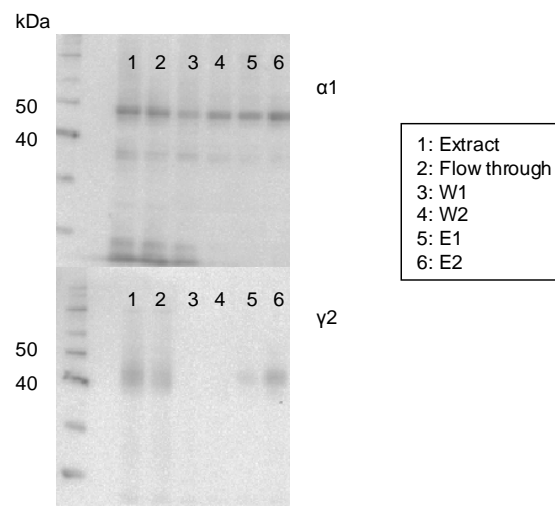


Fig. 40: SDS-PAGE and Western blot of the $\alpha_1\beta_3\gamma_2$ His12 receptor purification with a Ni-NTA spin column. Sf9 cells were co-infected with the $\alpha_1\beta_3\gamma_2$ His12 virus. Membranes and extracts were prepared and receptors purified via a Ni-NTA column. Aliquots from different washing (W1-W2: 40mM imidazole) and elution steps (E1-E2: 250mM imidazole) were precipitated, subjected to an SDS-PAGE and Western blot. The blots were stained with α_{1L} and γ_{2L} specific antibodies.

In the Western blot the α_1 subunit was detected in all samples at the apparent molecular mass of 50kDa. The α_1 antibody stained cross-reactivities at the molecular mass of 20, 25 and 35kDa as well. The γ_2 subunit was only detected in the extract (lane1), the flow through (lane 2) and in both eluates (lane 5 and 6) at the apparent molecular mass of 48kDa.

These results indicated that only small amounts of $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptors were expressed, that were only detectable with a Western blot (Fig. 40), but not in Coomassie blue staining (Fig. 39). This implied the necessity to use more material for the next purification experiment and the requirement of a minimum of expressed receptors for adequate purification.

3.3.4 Purification with a Ni-NTA column

A pellet of 510ml harvested Sf9 cells, expressing $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptors was used. This pellet contained 79pmol [³H]flunitrazepam binding sites corresponding to 20 μ g receptor protein.

Membranes were prepared from the Sf9 pellet (2.4.1), extracted in 120ml 0.5% Triton X-100 extraction buffer (2.4.2) and the clear supernatant applied to a 750 μ l Ni-NTA agarose column. The column was washed with 4ml of a 40mM imidazole buffer and then with 3 x 700 μ l aliquots of an 80mM imidazole buffer. The proteins were eluted with 5 x 700 μ l 250mM imidazole buffer aliquots (eluate 1-5). The samples were tested with SDS-PAGE, Coomassie blue staining (Fig. 41) and Western blot analysis (Fig. 42). In addition a [³H]flunitrazepam PEG precipitation assay was performed from the washing and the first 3 elution samples (Tab. 10).

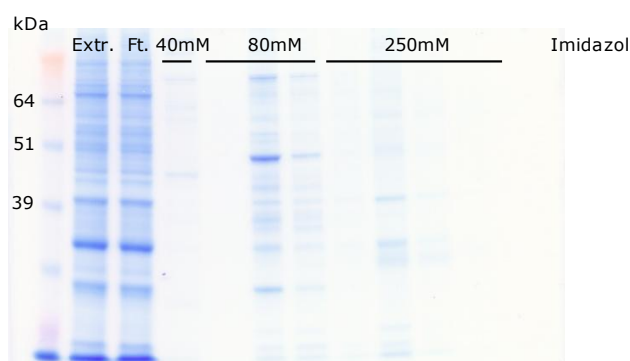


Fig. 41: SDS-PAGE and Coomassie blue staining of $\alpha_1\beta_3\gamma_2$ His12 receptors purification with a Ni-NTA column. Sf9 cells were infected with the $\alpha_1\beta_3\gamma_2$ His12 virus. Membranes and extracts were prepared and receptors purified via a Ni-NTA column. Aliquots from different washing and elution steps were precipitated, subjected to SDS-PAGE and stained with Coomassie blue.

The Coomassie blue staining showed no signals for the purified $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptor subunits. The α_1 , the β_3 and the γ_2 His12 subunits should exhibit a protein band at the apparent molecular mass of 50, 50 and 48kDa, respectively. All bands, stained in the Coomassie blue staining were unspecific proteins from the

extract. The strong band at about 48kDa, seen in the washing step with 80mM imidazole, was already detected in previous experiments and did not represent a GABA_A receptor subunit (Fig. 18). An adequate purification level was not reached with the low expression level of receptors.

To confirm that at least small amounts of α_1 , β_3 and γ_2 His12 were expressed and purified, a Western blot was performed with the same samples (Fig. 42).

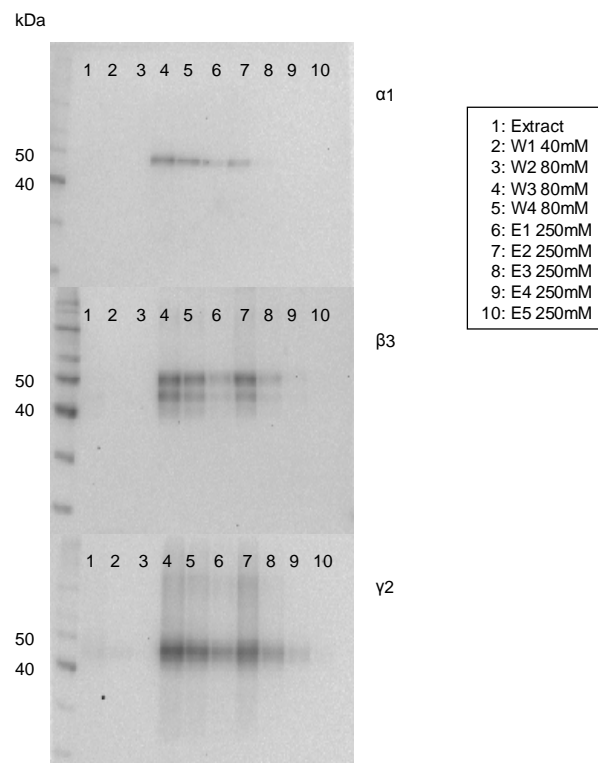


Fig. 42: SDS-PAGE and Western blot of $\alpha_1\beta_3\gamma_2$ His12 receptors purification with a Ni-NTA column. Sf9 cells were infected with the $\alpha_1\beta_3\gamma_2$ His12 virus. Membranes and extracts were prepared and receptors purified via a Ni-NTA column. Aliquots from different washing (W1: 40mM; W2-W4: 80mM imidazole) and elution steps (E1-E2: 250mM imidazole) were precipitated, subjected to an SDS-PAGE and Western blot. The blots were stained with α_{1L} , β_{3N} and γ_{2L} specific antibodies.

In the Western blot the α_1 , β_3 and γ_2 His12 subunits were stained at the apparent molecular mass in the washing (80mM imidazole) and elution (250mM imidazole) samples. The α_1 (50kDa) and the γ_2 His12 (48kDa) subunits indicated no degradation, whereas the β_3 subunit (50kDa) showed degraded protein bands at the molecular mass of 45kDa. This degradation could have caused the low expression level. Functionally assembled GABA_A receptors need intact β subunits.

This result indicated that the $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptors were already eluted from the column, starting with 80mM imidazole. At this low amount of imidazole, other

proteins from the extract, bound to the Ni-NTA column, were eluted as well. This problem resulted from the His-tag on the γ subunit, which is present in an $\alpha_1\beta_3\gamma_2$ receptor only once. The double His6-tag in the $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptors could not enhance the binding affinity to the Ni-NTA column. A His-tag on the β_3 subunit, which is present twice in an $\alpha_1\beta_3\gamma_2$ receptor, resulted in a stronger binding of the receptor to the column. Therefore the column could be washed more stringently and the receptor was eluted at a higher imidazole concentration (Fig. 18).

	pmol receptor [³ H]flunitrazepam/ 700µl
Washing 1	0.0
Washing 2	5.6
Washing 3	3.8
Eluate 1	1.7
Eluate 2	3.7
Eluate 3	0.7

Tab. 10: One-step purification of $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptors with a Ni-NTA column. Sf9 cells were infected with the $\alpha_1\beta_3\gamma_2$ His12 virus. Membranes and extracts were prepared and receptors purified via a Ni-NTA column. Samples from different stages of the purification (washing (W1: 40mM; W2-W4 80mM: imidazole and elution steps E1-E2: 250mM imidazole) were incubated with 10nM [³H]flunitrazepam \pm 100µM diazepam and specific binding (pmol [³H]flunitrazepam) was determined.

The performed [³H]flunitrazepam binding assay data (Tab. 10), using the same washing and elution samples, confirmed the results from the Coomassie blue staining (Fig. 41) and the Western blot (Fig. 42). Most of the $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptors were eluted with 80mM imidazole (washing 2 and 3). At this low imidazole concentration other bound proteins from the extract to the Ni-NTA column were eluted as well and thereby contaminating the receptor samples for further structural analysis.

3.3.5 Expression and characterization of $\alpha_1\beta_3\gamma_2$ His12 receptors plus additional β_3

The low expression level and the degraded β_3 subunit led to the assumption that the β_3 subunit was not expressed in the correct manner in the triple construct to form functional $\alpha_1\beta_3\gamma_2$ -containing receptors in Sf9 cells. Therefore in the next expression experiments, the triple construct viruses were co-infected with additional β_3 viruses in Sf9 cells.

Membranes from harvested Sf9 cells were prepared (2.3.1) and a radioligand binding assays with 10nM [3 H]flunitrazepam (2.3.3) was performed (Tab. 11). Sf9 cells were harvested after 2 days. In addition to the binding assay experiments, the samples were also tested for degradation of receptors with SDS-PAGE and Western blot analysis (2.6) (Fig. 43).

Additional β_3 viruses	dpi	pmol [3 H]flunitrazepam/mg total protein
500 μ l	2	0.4
1000 μ l	2	0.5
1500 μ l	2	0.5

Tab. 11: [3 H]flunitrazepam binding assay of an $\alpha_1\beta_3\gamma_2$ His12 plus additional β_3 virus expression. Sf9 cells were co-infected with a constant amount of $\alpha_1\beta_3\gamma_2$ His12 virus and increasing amount of β_3 virus and harvested after 2 days. Membranes and extracts were prepared and incubated with 10nM [3 H]flunitrazepam \pm 100 μ M diazepam and specific binding (pmol [3 H]flunitrazepam) was calculated.

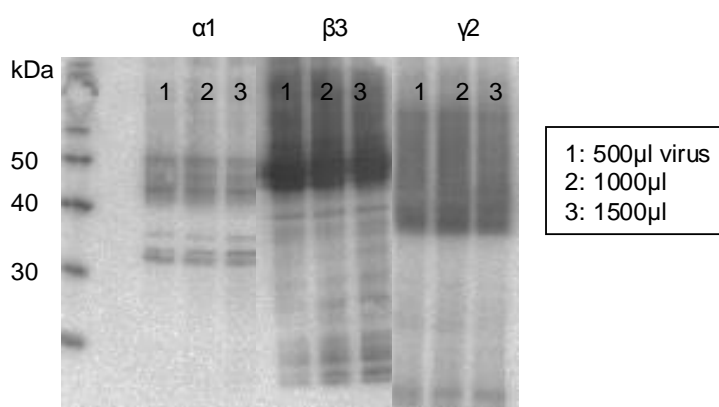


Fig. 43: SDS-PAGE and Western blot of an $\alpha_1\beta_3\gamma_2$ His12 plus additional β_3 viruses expression. Sf9 cells were infected with a constant amount of $\alpha_1\beta_3\gamma_2$ His12 virus and increasing amount of β_3 virus. Membranes were prepared and the same amount of total protein was subjected to an SDS-PAGE and Western blot. The blots were stained with α_{1L} , β_{3N} and γ_{2L} specific antibodies.

This binding assay experiment (Tab. 11) indicated that the expression level of the $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptor could not be enhanced by additionally added β_3 viruses. The same expression level of 0.5pmol [3 H]flunitrazepam binding/mg total protein was observed under the conditions where the $\alpha_1\beta_3\gamma_2$ His12 triple construct virus was used alone (Tab. 9).

SDS-PAGE and Western blot experiments (Fig. 43) indicated that the β_3 subunit was much stronger expressed due to the additional recombinant β_3 viruses, but the expression levels of the α_1 and γ_2 subunits were not enhanced in the Sf9 membrane

(compare with Fig. 38). This showed that the cells were infected with the additional β_3 viruses but instead of assembling more $\alpha_1\beta_3\gamma_2$ receptors, homooligomeric β_3 receptors were expressed.

The α_1 subunit at the apparent molecular mass of 50kDa was degraded. The degradation products were stained at 45 and 48kDa. The bands at around 35kDa were cross-reactions of the antibody. The strong signal of the β_3 subunit at 50kDa showed no degradation, but the β_3 antibody showed cross-reactivities between 15 and 40kDa. Most of the γ_2 subunits were degraded and stained at the apparent molecular mass of 40kDa, whereas the non-degraded γ_2 at 48kDa could be hardly detected. Bands at higher molecular masses indicated aggregates of the subunits, which were not dissolved properly during SDS-PAGE.

By co-infecting Sf9 cells with the $\alpha_1\beta_3\gamma_2$ His12 triple construct and β_3 alone it seemed that all three subunits showed stronger degradation in the Western blot (Fig. 43) compared to the $\alpha_1\beta_3\gamma_2$ His12 triple construct alone (Fig. 40).

For further structural analysis an expression of a minimum of 2 picomol pure receptor/mg protein is required. This is the approximate value of the amount of existing GABA_A receptors in the brain. At the moment this expression value could not be reached with the triple construct virus, expressing $\alpha_1\beta_3\gamma_2$ His12 GABA_A receptors.

3.4 Future prospects

The three different experimental setups to express and purify large amounts of $\alpha_1\beta_3\gamma_2$ GABA_A receptors indicated that the first approach with α_1 , β_3 His8 and γ_2 recombinant viruses and a two-step purification resulted in the largest amount of highly purified $\alpha_1\beta_3\gamma_2$ receptors (Fig. 19). However, using these conditions only 3% of the generated receptor could be purified. It is thus questionable whether the method can be up-scaled sufficiently to obtain large amounts of purified receptor required for crystallization trials.

Nevertheless large scale expression of this virus combination in Sf9 cells is currently performed for a different purpose. After purification, the receptor will be separated on a BN/SDS-PAGE. The group of Professor Lubec, Vienna is going to pick spots from the gel, make an in-gel digestion and identify the proteins and their sequence with qTOF. The sequence of the alpha1 and the beta3 subunit has already been published with this method (Kang et al. 2008; Kang and Lubec 2009). Therefore this group will concentrate on sequencing the gamma2 subunit.

Knowing also the sequence of the gamma2 GABA_A receptor subunit, it is planned to investigate drug-binding sites at the $\alpha_1\gamma_2$ interface of these receptors, identified by photoaffinity labeling of the receptors with suitable drugs. In addition, it is planned to investigate whether purified $\alpha_1\beta_3$ His8 γ_2 GABA_A receptors could be used for surface plasmon resonance studies performed by a collaborating group.

4 DISCUSSION

GABA_A receptors are targets for many clinically important drugs that influence excitability of the brain, motor function, anxiety, cognition, vigilance and memory (Sieghart 1995). The exact molecular structure of the GABA_A receptor, however, is still unknown, as it is the case for most receptors of the cys-loop receptor super-family.

Aim of this thesis was to produce recombinant hetero-oligomeric (alpha1-beta3-gamma2) GABA_A receptors for subsequent structural analysis, such as crystallization, photo affinity labeling or surface plasmon resonance investigations.

The $\alpha_1\beta_3\gamma_2$ receptors were expressed in the baculovirus expression system, which has become one of the most widely used systems for routine production of recombinant proteins. The baculovirus expression system has the advantages of eukaryotic posttranslational modifications and an easy and safe to use virus and cell culture system.

This thesis is based on the results of the thesis “High yield expression and His-tag purification of recombinant GABA_A receptors using Sf9-baculovirus Expression Vector System” from Leila Wabenegger. Single-construct viruses used in my thesis were already cloned and had to be amplified to working stocks and characterized with virus PCR and virus plaque assay experiments.

It was already published that expression of GABA_A receptors consisting of a single α_1 , β_1 or γ_2 subunit or dual combinations of $\alpha_1\gamma_2$, $\beta_1\gamma_2$ or $\beta_3\gamma_2$ did not lead to the formation of functional ion channels or binding sites (Hartnett et al. 1996). Functional homo-oligomeric GABA_A receptors can only be formed by the β_3 subunit. This receptor type has a binding pocket for several insecticides, such as α -endosulfan or lindane and can be detected with [³H]EBOB binding studies (Ratra and Casida 2001; Kang and Lubec 2009). Sf9 cells infected with α_1 and β_3 or α_1 , β_3 and γ_2 subunits produced homo-oligomeric β_3 , recombinant hetero-oligomeric $\alpha_1\beta_3$ or $\alpha_1\beta_3\gamma_2$ GABA_A receptors. The GABA binding pocket between an alpha and a beta subunit was measured with [³H]muscimol, whereas the benzodiazepine binding pocket between a beta and a gamma subunit was measured with a [³H]benzodiazepine, such as Ro15-1788 or flunitrazepam. These binding assay results also demonstrated that GABA_A receptors were functionally expressed by the baculovirus expression system (Hartnett et al. 1996).

In this thesis three different $\alpha_1\beta_3\gamma_2$ receptor types, which differ in the subunit composition, the additional His-tag and in the amount of baculovirus required to infect a single cell with all three subunits, were expressed, purified and characterized. The first experiments were performed with α_1 , β_3 His8, and γ_2 viruses. For the second co-infection α_1 , β_3 and γ_2 His8 viruses were used and the third receptor type was formed in Sf9 cells infected with a single virus containing the α_1 , β_3 , and γ_2 His12 subunits.

For large scale expression experiments huge amounts of viruses were needed. Therefore the existing virus stocks of α_1 , β_3 His8, γ_2 and γ_2 His8 viruses were scaled up to working stocks and the virus titer was determined with virus plaque assay experiments. The virus stock had to reach a minimum of 1×10^7 viruses/ml serum to be stable during storage and to infect Sf9 cells with a reliable probability. This meant a series of amplification experiments to obtain the best virus titer for each used virus.

In the first experiments α_1 , β_3 His8 and γ_2 viruses were used for co-infection of Sf9 cells to form $\alpha_1\beta_3\gamma_2$ GABA_A receptors. The ideal harvesting time point of the Sf9 cells was determined by Western blot analysis and [³H]flunitrazepam binding assays, presenting a high expression level of the receptors between 36 hours (1.5 days past infection (dpi)) and 72 hours (3dpi). This result confirmed the expression of the subunits under a late promoter. It seemed that the expression level of functional receptors could not be additionally enhanced by time, whereas the intensity of the degradation could not be diminished by harvesting the cells at a much earlier time point.

Knowing the appropriate harvesting time point, the best ratio of α_1 to β_3 His8 to γ_2 viruses and the multiplicity of infection (moi) were investigated with [³H]flunitrazepam binding assays. The used ratios of 2:1:1 and 3:1:1 and the moi of 1 and 2 showed no significant difference in the receptor expression level. Approximately 2pmol $\alpha_1\beta_3\gamma_2$ GABA_A receptors were expressed. For further expressions a ratio of 2:1:1 or 3:1:1 with a moi of 1 and a harvesting time point of 72 hours (3dpi) were used.

After optimization of expression and extraction conditions, $\alpha_1\beta_3\gamma_2$ receptors could be purified with a Ni-NTA column via the His-tag on the β_3 subunit. Approximately 30% of the applied receptors were eluted. [³H]flunitrazepam and [³H]muscimol binding assays indicated a disequilibrium of $\alpha_1\beta_3\gamma_2$ to $\alpha_1\beta_3$ -containing receptors. Only about 10% of the expressed GABA_A receptors contained a γ subunit. On the one hand the purification indicated a loss of 2/3 of the receptor but on the other hand 1/3 of the receptor could be purified quite well and concentrated in a small volume. Binding assays and a Western blot and Coomassie blue staining indicated that more β_3 His8

homo-oligomeric and $\alpha_1\beta_3$ His8 hetero-oligomeric receptors than the wanted $\alpha_1\beta_3$ His8 γ_2 GABA_A receptors were expressed and subsequently purified with the Ni-NTA column.

This heterogeneous mixture of receptors cannot be used for crystallization experiments. To avoid enrichment of β_3 His8 homo-oligomeric and $\alpha_1\beta_3$ His8 hetero-oligomeric receptors and to enhance the purity of $\alpha_1\beta_3$ His8 γ_2 receptors a two-step purification with a benzodiazepine affinity column and a subsequent Ni-NTA column was performed with a large amount of extracted proteins. The benzodiazepine column retained all alpha- and gamma-containing receptors, which were concentrated with the second column afterwards. Coomassie blue staining from the eluates after the benzodiazepine and the Ni-NTA column indicated a well stained, concentrated receptor with an estimated purity of 90%. This two-step purification, compared to the single Ni-NTA column purification, resulted again in a large loss of receptor. From the applied 700 μ g receptor protein, 20 μ g of pure receptor protein were eluted at the end. In principle, this pure receptor could be used for further analysis, but the amount was quite low.

Purification with two columns required a large amount of infected Sf9 cell material to obtain a useable, pure and functional amount of receptor at the end of the purification for further structural analysis. To overcome this problem a new series of co-infection of Sf9 cells was started. This time the His-tag was attached to the γ_2 subunit. The Sf9 cells, infected with α_1 , β_3 and γ_2 His8 baculoviruses should assemble and express $\alpha_1\beta_3\gamma_2$ His8 GABA_A receptors that then could be purified via the Ni-NTA column. Even if β_3 homo-oligomeric and $\alpha_1\beta_3$ hetero-oligomeric receptors were also assembled and expressed, they could not bind to the Ni-NTA column, thus the binding sites at the column should be occupied with $\alpha_1\beta_3\gamma_2$ His8 receptors only.

Once again the best parameters for the expression had to be determined. In several experiments the ratio of α_1 to β_3 to γ_2 His8, different multiplicities of infection (moi) and the best harvesting time point (dpi) were tested. [³H]flunitrazepam and [³H]muscimol binding assays indicated very low expression levels and the presence of a heterogeneous mixture of $\alpha_1\beta_3$ and $\alpha_1\beta_3\gamma_2$ GABA_A receptors. The expression did not change when different virus ratios, moi or harvesting time points were used. The [³H]flunitrazepam binding was around 0.5pmol/mg total protein.

Western blot analysis also showed no significant differences in the intensity of the bands at the different virus ratios, moi and harvesting time points. The γ_2 subunit was degraded strongly under all conditions used. This degradation explained the low expression level of $\alpha_1\beta_3\gamma_2$ His8 receptors, because a lack of functional γ_2 could lead to

an increase of $\alpha_1\beta_3$ and β_3 receptors. However, from these results it was concluded that a ratio of 3:1:1 with a moi = 1 and harvesting after 2dpi = 50h should be used for large scale expression.

Under the assumption that the degraded γ_2 subunit might not have assembled to intact GABA_A receptors, we decided to start purification experiments with large amounts of extracted receptors. After optimization of expression and extraction conditions for the $\alpha_1\beta_3\gamma_2$ His8 receptors, they could be purified by a Ni-NTA column via the His-tag on the γ_2 subunit. [³H]flunitrazepam binding assays showed a very low yield of GABA_A receptors. Almost the total amount of receptors was lost during washing of the Ni-NTA column. From 82pmol applied receptor protein, 15pmol were retained and 2.3pmol were eluted. Thus, the column retained approximately 20% of the applied receptor protein, but only 3% were eluted after washing. The low amount of eluted receptor could not be identified clearly in the Coomassie blue staining, but it could be detected by Western blot analysis, showing less degradation of the eluted receptors.

The use of γ_2 His8 baculoviruses showed a reduced overall expression of the receptor and no improvement in the large scale purification via a Ni-NTA column, compared to receptors containing the His-tag in the β_3 subunit. The degradation of the γ_2 subunit further diminished the assembly and expression of functional $\alpha_1\beta_3\gamma_2$ -containing receptors in Sf9 cells. The process of membrane preparation, extraction and purification with a single Ni-NTA column led to very little recovery of $\alpha_1\beta_3\gamma_2$ GABA_A receptors in the end.

A low expression could also have been caused by the low probability that a single Sf9 cell was infected with all three subunits (α , β and γ viruses) to form functional $\alpha_1\beta_3\gamma_2$ receptors. To enhance the possibility that each cell was infected with all three subunits the next experimental approach was to clone all three subunits into one vector, to obtain a baculovirus able to infect a cell with all three subunits. In addition a His12-tag (two times 6 Histidines) was cloned into the γ_2 subunit to increase the binding strength of the receptor to a Ni-NTA column. This should provide the opportunity to wash the column with higher concentrations of imidazole.

All three subunits were cloned into a pBAC4x-1 vector. The plasmid contains two polh and two p10 promoters; each of which is upstream of unique cloning sites for sequential insertion of target genes. The homologous promoters are in opposite orientation to minimize recombination. In order to reduce the size of the plasmid for subsequent cloning steps, the promoter region of the plasmid pBAC4x-1 was cloned into pUC18. After sequential insertion of an α_1 , a β_3 and a γ_2 subunit the

completed insert was cloned back into the pBAC4x-1 vector, resulting in a 10242bp large triple construct.

For preliminary and large scale expression experiments a large amount of virus was needed. For that Sf9 cells were transfected with the triple construct to produce recombinant viruses. After reaching a functional initial virus stock of $\alpha_1\beta_3\gamma_2$ His12 viruses, they were scaled up to working stocks and the virus titer was determined with virus plaque assay experiments after every step.

When a certain amount of virus working stock was reached, preliminary experiments with this new virus were performed. The best expression parameters, meaning the moi and the harvesting time point, were determined in several expression experiments. [3 H]flunitrazepam and [3 H]muscimol binding assays indicated very low expression levels, below the picomolar range. The [3 H]flunitrazepam binding was approximately 0.5pmol and the [3 H]muscimol binding around 0.3pmol per mg total protein for all conditions. These values were approximately in the same range and implied that a lot of receptors contained a gamma subunit. Even though the expression level was very low, the fact that a lot of receptors contained a gamma subunit was very positive. In addition the binding of [3 H]flunitrazepam and [3 H]muscimol indicated functionally expressed receptors, containing the benzodiazepine as well as the GABA binding pocket. The expressed 0.5pmol $\alpha_1\beta_3\gamma_2$ His12 receptors were comparable with the expressed amount of $\alpha_1\beta_3\gamma_2$ His8 receptors. Additionally performed Western blots of the same samples indicated degradation of all three subunits. This degradation could have caused the low amount of functionally expressed $\alpha_1\beta_3\gamma_2$ His12 receptors.

To optimize extraction and purification conditions a small amount of the newly expressed $\alpha_1\beta_3\gamma_2$ His12 receptor was purified with a Ni-NTA spin column and a Coomassie blue staining and a Western blot of the washing and elution samples was performed. No enhanced bands at the expected molecular masses were stained. This preliminary experiment indicated that we were not able to purify the receptor with a Ni-NTA spin column with the His12-tag on the γ_2 subunit.

Although the determination of the optimal conditions showed degradation of the subunits in the membrane, we started purification experiments, hoping that only non-degraded, functional $\alpha_1\beta_3\gamma_2$ His12 receptors, with a non-degraded His-tag on the γ_2 subunit would be assembled and bound to the Ni-NTA column. By using a large scale expression we hoped that the amount of lost receptors could be balanced. Therefore a large batch of $\alpha_1\beta_3\gamma_2$ His12 receptors was purified with a Ni-NTA column. A Western blot of the washing and the elution samples showed a specific signal of all three

subunits at the appropriate molecular masses, starting at an imidazole concentration of 80mM. The β_3 specific antibody also stained degradation products. A [3 H]flunitrazepam binding assay of the same washing and elution samples indicated that most of the $\alpha_1\beta_3\gamma_2$ His12 receptor was eluted during washing with 80mM imidazole. With this low amount of imidazole not only receptors but also a lot of other proteins were eluted from the column, which was seen in a Coomassie blue staining of the same samples. The double 6 His-tag (His12) on the γ_2 subunit thus did not exhibit the expected strong binding to the Ni-NTA column, compared to a receptor with a His8-tag on the β_3 subunit, which exists twice in an $\alpha_1\beta_3\gamma_2$ receptor. Due to the 2 β_3 -His8 subunits, the $\alpha_1\beta_3$ His8 γ_2 GABA_A receptor could be eluted with 200mM imidazole and therefore the eluted fractions exhibited a much higher purity.

The low expression level and the β_3 degradation led to the assumption that the β_3 subunit was not expressed in the correct manner in the triple construct to form $\alpha_1\beta_3\gamma_2$ receptors in Sf9 cells. Thus further expression experiments were done with the triple construct and additional β_3 viruses for the infection of Sf9 cells. [3 H]flunitrazepam binding indicated no enhancement in the expression of $\alpha_1\beta_3\gamma_2$ receptors in the membrane. The [3 H]flunitrazepam binding reached the same level of 0.5pmol per mg total protein independent of increasing amounts of additional recombinant β_3 viruses. A Western blot with subunit specific antibodies confirmed this result and indicated degradation for all three subunits.

The degradation and the low expression level of the triple construct compared to the single viruses expressing $\alpha_1\beta_3$ His8 γ_2 GABA_A receptors, could be caused by the different ratios of the transfected subunits. Sf9 cells were transfected with the ratios of 2:1:1 and 3:1:1 to produce $\alpha_1\beta_3$ His8 γ_2 GABA_A receptors whereas the ratio of the expressed triple construct subunits is not known. In the triple construct the expression of the α_1 and the γ_2 subunit was under the control of the p10 promoter, whereas the β_3 His8 subunit was under the control of the polh promoter. Both promoters could differ in promoter activity and time point of activation leading to problems during the assembly of functional GABA_A receptors.

Summarizing the results for the triple construct, we showed that we were able to clone all three subunits of a GABA_A receptor into one vector and to produce recombinant baculoviruses that express very little, but functional receptors in Sf9 cells. The resulting receptors contain active GABA and benzodiazepine binding pockets.

In summary, three different approaches were investigated for the production and purification of large amounts of $\alpha_1\beta_3\gamma_2$ GABA_A receptors in this thesis. Experiments

indicated, that the first approach with α_1 , β_3 His8 and γ_2 recombinant viruses and a two-step purification yielded the purest $\alpha_1\beta_3\gamma_2$ receptors, but only 3% of total receptors could be purified. We were able to express the receptor in the picomolar range, which can be used for further structural analysis, but not for crystallization. The other two approaches yielded expression levels around 0.5pmol receptor/mg total protein and we were not able to concentrate these receptors with the Ni-NTA column, thus showing, that a minimum of expressed receptors in the membrane is needed for purification.

Nevertheless large scale expression of the α_1 , β_3 His8 and γ_2 recombinant virus combination in Sf9 cells is currently performed for a different purpose. After purification, the receptor will be separated on a BlueNative(BN)/SDS-PAGE. The group of Professor Lubec, Vienna is going to pick spots from the gel, make an in-gel digestion and identify the proteins and their sequence with qTOF. The sequence of the alpha1 and the beta3 subunit has already been published with this method (Kang et al. 2008; Kang and Lubec 2009). This group therefore will concentrate on sequencing the gamma2 subunit.

Knowing also the sequence of the gamma2 GABA_A receptor subunit, it is planned to investigate drug-binding sites at the $\alpha_1\gamma_2$ interface of these receptors identified by photoaffinity labeling of the receptors with suitable drugs. In addition, it is planned to investigate whether purified $\alpha_1\beta_3$ His8 γ_2 GABA_A receptors could be used for surface plasmon resonance studies performed by a collaborating group.

5 ABBREVIATIONS

AcMNPV	Autographa californica multiple nuclear polyhedrosis virus
APS	Ammoniumpersulfate
B _{max}	Maximal binding site density
BCA	Bicinchoninic acid
BZ	Benzodiazepine
CDS	Coding sequence
CNS	Central nervous system
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
Dpi	Days post infection
EDTA	Ethylenediaminetetraacetic acid
GABA	γ-Aminobutyric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
KCl	Kaliumchlorid
K _D	Ligand concentration where 50% of all receptors are occupied
kDa	Kilo Dalton
MBP	Maltose binding protein
Moi	Multiplicity of infection
MOPS	N-Morpholino propanesulfonic acid
Ni-NTA	Nickel-Nitrilotriacidic acid
PAGE	Polyacrylamide gel electrophoresis
Pfu	Plaque forming unit
PBS	Phosphate buffered saline
PMSF	Phenylmethanesulfonylfluoride
RNase	Ribonuclease
RT	Room temperature
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetra-methyl-ethylene-diamine
Tris	Tris (hydroxymethyl) aminoethane

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Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

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9 SUMMARY / ZUSAMMENFASSUNG

9.1 Summary

The γ -aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the mammalian central nervous system. GABA acts via two different classes of receptors, the GABA_A receptors, which are Cl⁻ channels that can be opened by GABA and the GABA_B receptors, which are indirectly coupled to Ca²⁺ and K⁺ channels via second messenger systems. GABA_A receptors are composed of five subunits that can belong to eight different subunit classes. The majority of receptors are composed of two α , two β and one γ subunits.

In addition to GABA, many drugs, such as benzodiazepines, barbiturates, steroids, anaesthetics, and convulsants, are able to bind to and thereby modulate the GABA_A receptors. The exact molecular structure is still unknown, as it is the case for most receptors of the cys-loop receptor super-family. Aim of this thesis was to produce and purify large amounts of recombinant hetero-oligomeric ($\alpha_1\beta_3\gamma_2$) GABA_A receptors for subsequent structural analysis, such as crystallization, photo affinity labeling or surface plasmon resonance investigations.

For that, receptors with three different subunit compositions ($\alpha_1\beta_3\text{His}8\gamma_2$, $\alpha_1\beta_3\gamma_2\text{His}8$ and $\alpha_1\beta_3\gamma_2\text{His}12$) were expressed using the baculovirus expression system in Sf9 cells for large scale purification. The $\alpha_1\beta_3\gamma_2\text{His}12$ construct was cloned newly into a pBac4x-1 vector. After generating stable baculovirus stocks for all constructs, the best expression conditions were determined for each receptor type using [³H]muscimol and [³H]benzodiazepine binding studies. Degradation of the proteins was investigated by SDS-polyacrylamide gel electrophoresis, Coomassie blue staining and Western blot analysis. Purification of the receptors via their His tag using a Ni-NTA column (and a benzodiazepine containing affinity column for the $\alpha_1\beta_3\text{His}8\gamma_2$ construct) provided dissimilar results. In a two-step purification the $\alpha_1\beta_3\text{His}8\gamma_2$ receptor resulted in an estimated purity of 90% with little degradation of the subunits, but with a low yield. The $\alpha_1\beta_3\gamma_2\text{His}8$ and the $\alpha_1\beta_3\gamma_2\text{His}12$ receptors presented a reduced overall expression with degradation products of the subunits and thus the recovery was lower as for the $\alpha_1\beta_3\text{His}8\gamma_2$ receptor. Since a minimum of functional receptors in the membrane is needed for purification, the expressed $\alpha_1\beta_3\text{His}8\gamma_2$ construct with a two-step purification will be used for further structural analysis.

9.2 Zusammenfassung

Die γ -Aminobuttersäure (GABA) ist die am häufigsten vorkommende inhibitorische Neurotransmitter-Substanz im zentralen Nervensystem von Säugetieren. GABA wirkt über zwei verschiedene Klassen von Rezeptoren, die GABA_A-Rezeptoren, die Cl⁻-Kanäle sind, welche durch GABA geöffnet werden können, und die GABA_B-Rezeptoren, die indirekt über *second messenger systems* an Ca²⁺- oder K⁺-Kanäle gekoppelt sind. GABA_A-Rezeptoren bestehen aus fünf Untereinheiten, die acht verschiedenen Untereinheitsklassen angehören können. Die Mehrheit der Rezeptoren besteht aus zwei α , zwei β und einer γ Untereinheiten.

Neben GABA können viele Wirkstoffe, wie Benzodiazepine, Barbiturate, Steroide, Anästhetika oder Konvulsiva, an GABA_A-Rezeptoren binden und diese modulieren. Wie bei den meisten Rezeptoren der *cys-loop receptor super-family*, ist die genaue Struktur des GABA_A-Rezeptors bis jetzt nicht bekannt. Ziel dieser Arbeit war die Produktion und Reinigung von großen Mengen rekombinanter hetero-oligomerer ($\alpha_1\beta_3\gamma_2$) GABA_A-Rezeptoren, die anschließend mittels Kristallisationsversuchen, *photo affinity labeling* oder *surface plasmon resonance* zur strukturellen Analyse verwendet werden sollten.

Zu diesem Zweck wurden drei verschiedene Untereinheits-Kombinationen ($\alpha_1\beta_3\text{His}8\gamma_2$, $\alpha_1\beta_3\gamma_2\text{His}8$ und $\alpha_1\beta_3\gamma_2\text{His}12$) im Baculovirus Expressionssystem in Sf9 Zellen exprimiert, um diese dann im großen Maßstab zu reinigen. Das $\alpha_1\beta_3\gamma_2\text{His}12$ Konstrukt wurde neu in einen pBAC4x-1 Vektor kloniert. Nachdem stabile Virenstocks für alle Kombinationen erzeugt wurden, wurden die besten Expressionsbedingungen für jeden Rezeptortyp mittels [³H]Muscimol und [³H]Benzodiazepin Bindungsstudien bestimmt. Der Abbau der Proteine wurde mittels *SDS-polyacrylamide gel electrophoresis*, *Coomassie blue staining* und *Western blot analysis* untersucht. Die Reinigung der Rezeptoren über deren His-Tag mittels einer Ni-NTA Säule (und einer Benzodiazepin beinhaltenden Affinitätssäule für den $\alpha_1\beta_3\text{His}8\gamma_2$ Rezeptor) ergab unterschiedliche Ergebnisse. Mittels 2-Stufenreinigung konnte der $\alpha_1\beta_3\text{His}8\gamma_2$ Rezeptor mit einer geschätzten Reinheit von 90% und geringem Abbau der Untereinheiten, allerdings mit sehr geringer Ausbeute, gereinigt werden. Die $\alpha_1\beta_3\gamma_2\text{His}8$ und $\alpha_1\beta_3\gamma_2\text{His}12$ Rezeptoren zeigten eine verminderte Expression und Abbauprodukte und somit eine geringere Ausbeute der Konstrukte als beim $\alpha_1\beta_3\text{His}8\gamma_2$ Rezeptor. Da für eine Reinigung ein Minimum an intakten Rezeptoren in der Membran vorhanden sein muss, wird in Zukunft das $\alpha_1\beta_3\text{His}8\gamma_2$ Konstrukt mit einer 2-Stufenreinigung für weitere Strukturanalysen verwendet werden.

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