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A novel open channel blocker of $GABA_A$ receptors

PhD Thesis submitted by

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Abstract

$GABA_A$ receptors are chloride ion channels composed of five subunits, mediating fast synaptic and tonic inhibition in the mammalian brain. 19 different subunit isoforms have been identified, with the major receptor type in mammalian adult brain consisting of α_1, β_2, and γ_2 subunits. $GABA_A$ receptors are the target of numerous sedating and anxiolytic drugs such as benzodiazepines. The currently known endogenous ligands are GABA, neurosteroids and the endocannabinoid 2-arachidonoyl glycerol (2-AG). The pharmacological properties of this chloride ion channel strictly depend on receptor subunit composition and arrangement. $GABA_A$ receptors bind and are inhibited by epileptogenic agents such as picrotoxin, and cyclodiene insecticides such as dieldrin. We screened aromatic monovalent anions with five-fold symmetry for inhibition of $GABA_A$ receptors. One of the anions, $PCCP^-$ inhibited currents elicited by GABA with comparable potency as picrotoxin. This inhibition showed all characteristics of an open channel block. The $GABA_A$ receptor ion channel is lined by residues from the M2 membrane-spanning segment. To identify important residues of the pore involved in the interaction with the blocking molecules $PCCP^-$, a mutation scan was performed in combination with subsequent analysis of the expressed mutant proteins using electrophysiological techniques.

In a second project we characterised a light-switchable modulator of $GABA_A$ receptors based on propofol. It was my responsibility to investigate the switching kinetics in patch clamp experiments. After its discovery in 1980, propofol has become the most widely used intravenous general anaesthetic. It is commonly accepted that the anaesthesia induced by this unusually lipophilic drug mostly results from potentiation of GABA induced currents. While $GABA_A$ receptors respond to a variety of ligands, they are normally not sensitive towards light. This light sensitivity could be indirectly achieved by using modulators that can be optically switched between an active and an inactive form. We tested an azobenzene derivative of propofol where an aryldiazene unit is directly coupled to the pharmacophore. This molecule was termed azopropofol (AP2). The effect of AP2 on Cl- currents was investigated with electrophysiological techniques using $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors expressed in Xenopus oocytes and HEK-cells.

In the third project we wanted to investigate the functional role of $GABA_A$ receptors in the liver, and their possible involvement in cell proliferation. $GABA_A$ receptors are also found in a wide range of peripheral tissues, including parts of the peripheral nervous system and non-neural tissues such as smooth muscle, the female reproductive system, liver and several cancer tissues. However their precise function in non neuronal or cancerous cells is still unknown. For this purpose we investigated expression, localization and function of the hepatocytes $GABA_A$ receptors in model cell lines and healthy and cancerous hepatocytes.

To my grandparents…..

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Abbreviations

CNS	central nervous system
PNS	peripheral nervous system
IPSPs	inhibitory postsynaptic potentials
EPSPs	excitatory postsynaptic potentials
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
VGAT	vesicular GABA transporter
GAT	GABA transporter
GABAx	γ-aminobutyric acid type x receptor
5-HT3	5-hydroxy-tryptamine type 3
N/P/Q-type Ca2+ channels	voltage dependent Calcium channels
GIRK channels	G protein-coupled inwardly-rectifying potassium channel
Cl^-	chloride ions
Ca^{2+}	calcium ions
TM	transmembrane domain
BiP	heavy-chain-binding protein
GluCl channel	glutamate-gated chloride channel
$PCCP^-$	pentacyanocyclopentdienyl anion
$MTSET^+$	(2-(trimethylammonium) ethyl methanethiosulfonate
AP2	azopropofol
RDL	Drosophila melanogaster GABA receptor
HEPES	2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethansulfonic acid
EGTA	ethylene glycol tetraacetic acid
PMSF	phenylmethanesulfonyl fluoride
PBST	Phosphate Buffered Saline solution with Tween® 20
IHH	immortalized human hepatocyte cell line
MRP	multidrug resistance protein

1 Introduction

1.1 Central nervous system

The nervous system can be divided into two parts: The central nervous system (CNS) is the part of the nervous system consisting of the brain and spinal cord. It is opposed to the peripheral nervous system (PNS), which is composed of nerves leading to and from the CNS throughout the rest of the body. The nervous system sends and receives information from all parts of the body, regulating internal organ function and responding to changes in the external environment.

The human body is made up of trillions of cells. The human brain has approximately 86 billion neurons (Azevedo et al., 2009) and the rest is formed by three different types of non neuronal cells called glia cells. The main functions of the glia cells are to support and provide nutriments and oxygen for the neurons, to control the communication between neurons at the synapsis; they are also responsible of the immune response, cleaning up the CNS from damaged neurons and infectious agents. (Allen and Barres, 2009; Araque and Navarrete, 2010). A typical neuron, different from the other cells, has specialized cell parts called dendrites and axon, which enable them to send and receive information**.** Dendrites bring electrical signals to the cell body and axons take information away from the cell body. Neuronal signalling actually involves both electrical and chemical or processes. Communication between neurons is achieved at synapses and it is called neurotransmission. To achieve long distance and rapid communication, neurons have evolved special abilities for sending electrical signals along axons in the form of an action potential (**Figure 1**). The neuron has a resting potential around -70 mV with the inside of the neuron negatively charged relative to the outside the cell. The resting potential of the neuron refers to the difference between the voltage inside and outside the neuron. The plasma membrane of a neuron contains voltage-gated cation channels, which are responsible for generating the action potentials.

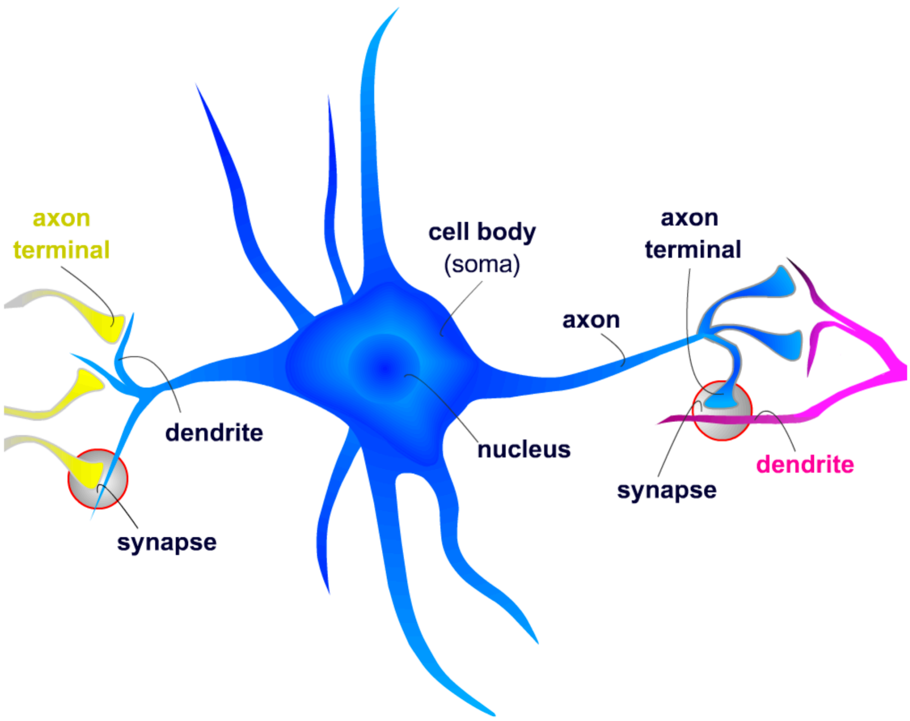

Figure 1. Neuronal signalling. (figure taken from Elliott , 2012.)

A stimulus sent out from a cell body, that causes sufficient depolarization promptly opens the voltage-gated Na^+ channels, allowing Na^+ ions to enter the cell down their electrochemical gradient causing a depolarization of the membrane. Once the cell reaches a certain threshold, an action potential will fire, sending the electrical signal down the axon. After the neuron has fired the potassium channels open, allowing flow of K^+ ions inside the cell and the sodium channels close, gradually returning the neuron to its resting potential, this is called “refractory period” in which another action potential is not possible. In myelinated axons, the sodium channels are present only at the nodes of Ranvier, the only place where the signal can be propagate along the excitable axon (**Figure 2**). The axonal membrane will be depolarized from one node to the other; this form of impulse propagation is called saltatory conduction. Such movement of the wave of depolarization is much more rapid than in unmyelinated fibers.

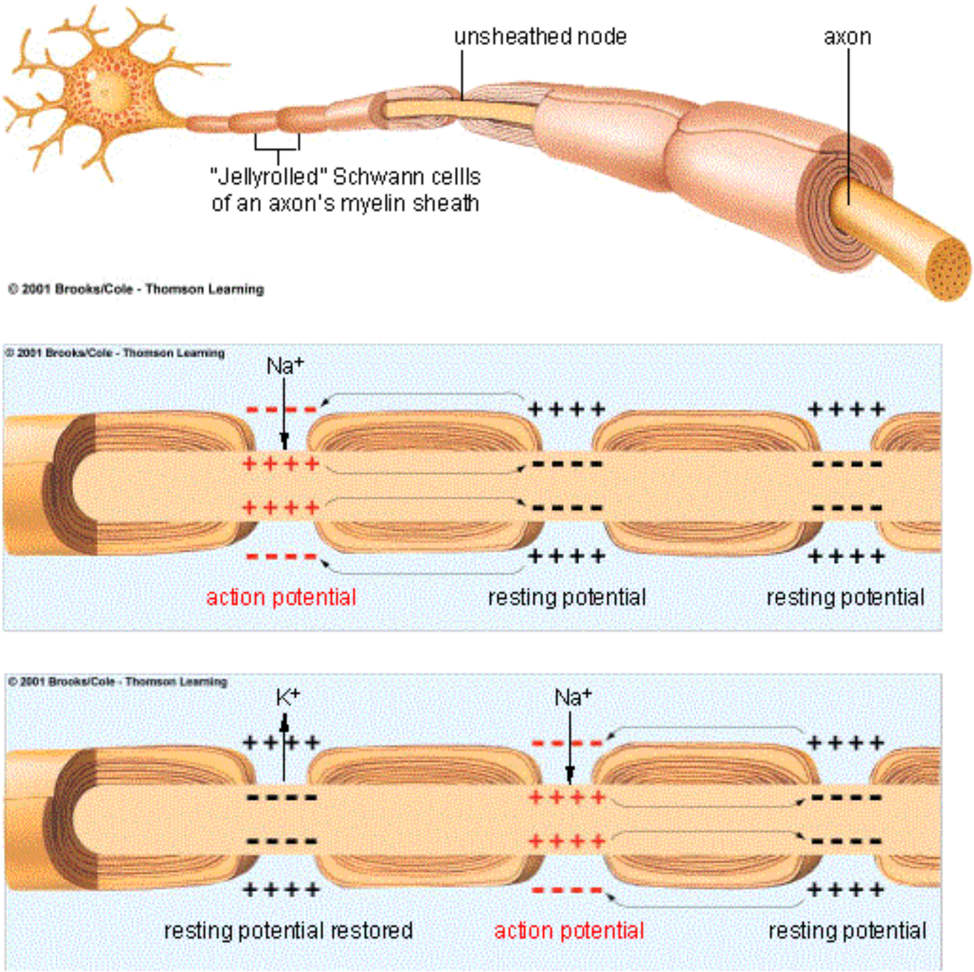

Figure 2. Representation of the propagation of an action potential along the axon. (figure taken from Thomson Learning, 2001).

1.1.1 Excitatory and inhibitory synapses

The contacts between the dendrites of one neuron with other neurons are termed synapses. Each neuron forms thousands of synapses with other neurons or other types of cells. In almost all the synapses, transmission is in one direction from the first (or presynaptic) neuron to the second or (postsynaptic) neurons. An arriving action potential depolarizes the pre-synaptic neuron and opens voltage-dependent calcium channels. The influx of calcium ions increases the calcium concentration in the pre-synaptic terminal, which in turn leads the vesicle containing the neurotransmitters to fuse with the plasma membrane and release the neurotransmitters into the synaptic cleft. The small volume of the cleft allows neurotransmitter concentration to be raised and lowered rapidly. In order to have any effect on the postsynaptic cell, a neurotransmitter molecule must fit onto a receptor precisely as there are specific receptors for specific neurotransmitters.

There are two major types of neurotransmitter receptors. The first type consistes of ion channels. These receptors opens after binding of neurotransmitter molecules and act in the millisecond time range (fast neurotransmission). The second type consistes of G-protein coupled receptors. These are coupled to second messenger system and act in the 100 milliseconds time range (slow neurotransmission). Most neurotransmitters interact primarily with post-synaptic receptors, but there are also presynaptic receptors, which provide fine control of neurotransmitter release by a negative feedback mechanism.

In the fast neurotransmission when a neurotransmitter binds to a receptor and leads to the opening of the integral ion channel, which results in a change in the membrane potential of a postsynaptic cell. Synaptic activation of inhibitory or excitatory neurotransmitter receptors respectively generate an inhibitory postsynaptic potential (IPSPs) or excitatory postsynaptic potentials (EPSPs). The most common inhibitory neurotransmitters in the nervous system are γ-aminobutyric acid (GABA) and glycine; the most abundant excitatory neurotransmitters are glutamate or acetylcholine.

A postsynaptic potential is termed as inhibitory when the resulting change in membrane voltage makes it more difficult for the cell to fire an action potential, lowering the firing rate of the neuron. They are the opposite of excitatory postsynaptic potentials, which result from the flow of ions like sodium into the cell because the opening of postsynaptic cation channels depolarizes the postsynaptic nerve terminal and trigger a new action potential in the post-synaptic cell.

1.2 GABAergic neurotransmission

1.2.1 Biosynthesis and metabolism of GABA

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system of mammals (Curtis et al., 1995; Mekarnan and Whithing, 1996; Krnjevic, 1997). About 20–50% of all synapses utilize this neurotransmitter (Barnard et al., 1998; Mehta and Ticku, 1999). **Figure 3** summarizes synthesis, release, and reuptake of GABA.

Two different forms of the enzyme glutamic acid decarboxylase (GAD) (GAD65 and GAD67) catalyse the decarboxylation of glutamate to GABA at the synaptic terminals. Most of the GABA and glutamate derive from the reserves of glutamine present in the glial cells. The main source of glutamine and glutamic acid, and then GABA, is glucose: in fact, in the Krebs cycle one of the products of the glucose metabolism is the α-ketoglutaric acid, which is converted to L-glutamic acid by the enzyme transaminase. After the synthesis, GABA neurotransmitter is transported to the synaptic vesicles through a vesicular neurotransmitter transporter (VGAT) and released by a calcium-dependent exocytosis mechanism.

The physiological effect by GABA is mediated by the action of GABA on ionotropic or metabotropic receptors, located in the pre- and post-synaptic terminals. The synaptic action of GABA is terminated by the action of the reuptake proteins of the neurotransmitter (GATs), which are located in the cytoplasmic membranes of the nerve terminals and in the surrounding glial cells (astrocytes). GABA-transaminase present in the mitochondria of the glial cells and neurons, catalyses the conversion of GABA into succinic semialdehyde and glutamate. Succinic semialdehyde is then oxidised into succinic acid by succinic semialdehyde dehydrogenase and as such enters the citric acid cycle as a usable source of energy.

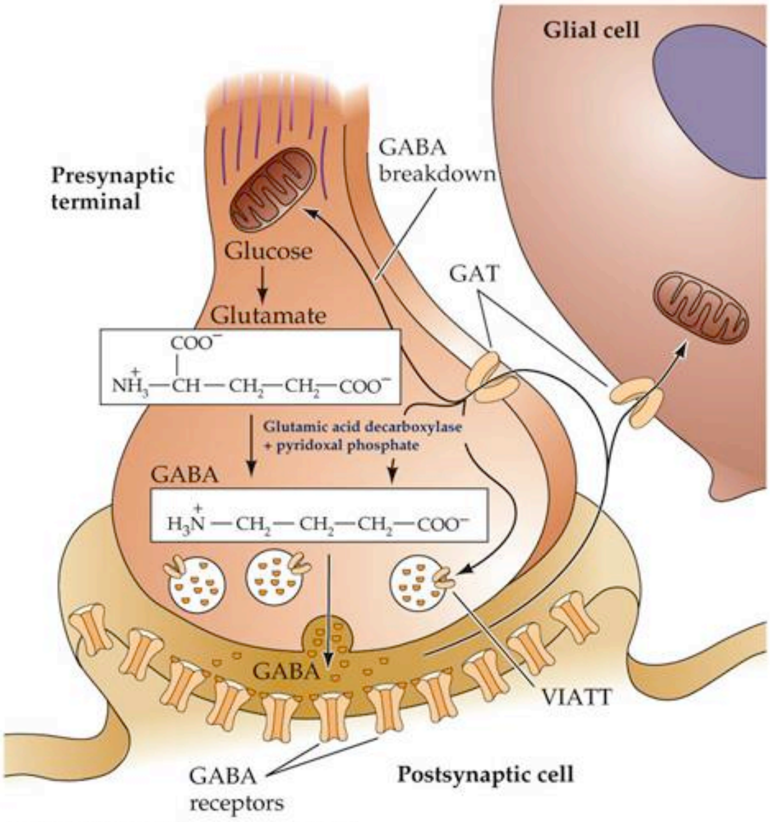

Figure 3. Schematic representation of the synthesis, release, and reuptake of the inhibitory neurotransmitter GABA. (figure taken from Purves D. et al. 2001).

It is estimated that more than 90% of all GABA in the mammalian CNS is degraded in this way and contributes to energy metabolism in the citric acid cycle.

The GABAergic function is finely regulated at multiple levels (Cherubini and Conti, 2001) which includes the synthesis of the neurotransmitter from the two isoforms of the glutamic acid carboxylase (GAD) (Erlander et al., 1991; Esclapez et al., 1994; Soghomonian and Martin, 1998); by the regulation of vesicular transporter which would be responsible for the storage of GABA in the synaptic vesicle and subsequent release at GABA synapses (Dumoulin et al., 1999; Gasnier, 2000), by the release of Ca^{2+} (Wall and Usowicz, 1997; Vautrin et al., 2000; Kirischuk et al., 2002), by reuptake into neurons and glial cells (Borden, 1996; Quick et al., 1997) and the activation of multiple receptors located pre-, post-, and extra-synaptically.

1.3 GABA receptors

1.3.1 Classification

GABA is capable of interacting with two major subtypes of specific receptors: ionotropic $GABA_A$ receptors (Barnard et al., 1998) and metabotropic $GABA_B$ receptors (Bowery et al., 2002) (**Figure 4**), which are expressed ubiquitously in the CNS. There is also another subclass of $GABA_A$ receptors, associated to ρ subunit, almost exclusively expressed in the retina (Bormann and Feingespan, 1995; Kusama et al., 1995; Chebib et al., 1997), previously known as the $GABA_C$ receptor. However since $GABA_C$ receptors are closely related in sequence, structure, and function to $GABA_A$ receptors the nomenclature committee of the IUPHAR has recommended that the use of $GABA_C$ name should be abandoned and these ρ receptors should be designated as the ρ subfamily of the $GABA_A$ receptors (Barnard et al., 1998; Olsen and Sieghart, 2008).

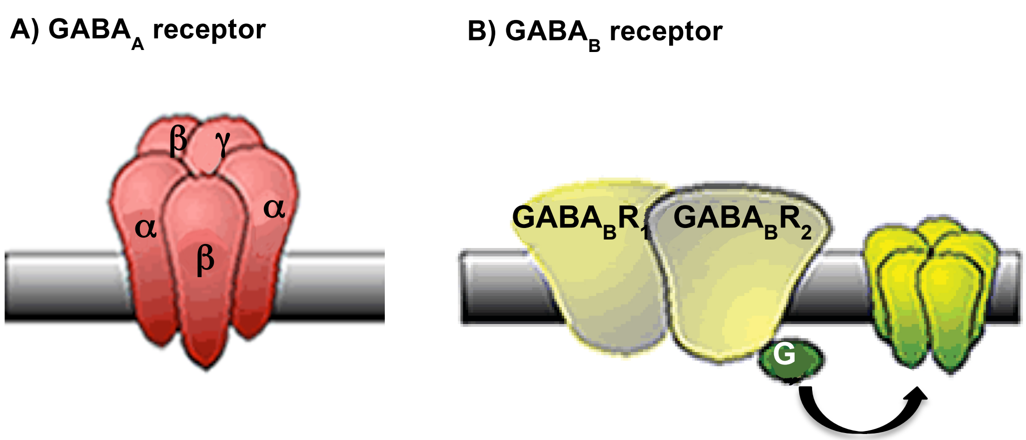

Figure 4. Schematic representation of the different subtypes of the GABA receptor. A) $GABA_A$ receptor, B) $GABA_B$ receptor.

1.3.2 $GABA_A$ Receptors

$GABA_A$ receptors belong to the family of cys-loop receptors characterized by a disulphide bond between two cysteine residues in the extracellular domain. This family consists of the nicotinic acetylcholine receptors, the $GABA_A$ and the 5-HT_3 receptors, the glycine receptors and some bacterial receptors (Macdonald and Olsen, 1994; Dunn et al., 1994; Rabow et al., 1995; Barnard et al., 1998; Hervers and Luddens, 1998; Enz, 2001; Sieghart and Sperk, 2002; Chebib, 2004; Gibbs and Johnston, 2005).

$GABA_A$ receptors are heterooligomeric receptors, constituted by the assembly of five different or identical subunits that form an ion channel permeable to chloride ions (Macdonald and Olsen, 1994; Sieghart, 1995; Sieghart and Sperk, 2002; Sigel and Steinmann, 2012) (**Figure 5**).

They are activated by two molecules of GABA interacting on their specific sites. The binding of GABA to the receptor leads to a rapid opening of the ion channel associated to the protein, through which chloride ions flow according to their electrochemical gradient. An increased neuronal chloride conductance leads to a reduction of the probability that an action potential can be initiated in this cell. $GABA_A$ receptors mediate fast synaptic inhibition but in few cases, these receptors have been reported to act excitatory, for example during nervous system development (Ben-Ari et al., 1997; Taketo and Yoshioka, 2000) or in certain cell populations (cells of pituitary pars intermedia, loci in embryonic and early postnatal life in the mammal, SP-O interneurons in the rat hippocampal CA3 area) (Tomiko et al., 1983; Cherubini et al., 1991; Ben-Ari et al., 1997; Lamsa and Taira, 2003). This exceptional phenomenon is a consequence of high internal Cl^- concentration in these neurons.

Due to their wide distribution within the nervous system of mammals, the $GABA_A$ receptors play a role in virtually all brain functions. The highest $GABA_A$ receptor densities are found in the brain cortex, followed by the hypothalamus, cerebellum, hippocampus and striatum (Braestrup et al., 1977; Fritschy and Möhler, 1995; McKernan and Whiting, 1996; Bateson, 2004). Receptor populations are also found in midbrain, medulla oblongatapons and spinal cord (Fritschy and Möhler, 1995; Bateson, 2004). As mentioned below, $GABA_A$ receptors can also be found, but in a limited amount, in non-neural tissues such as the pancreas, placenta, immune cells, liver, bone growth plates and several other endocrine tissue (Minuk et al., 2007) where their functional roles are still under study and their pharmacological relevance remains to be established.

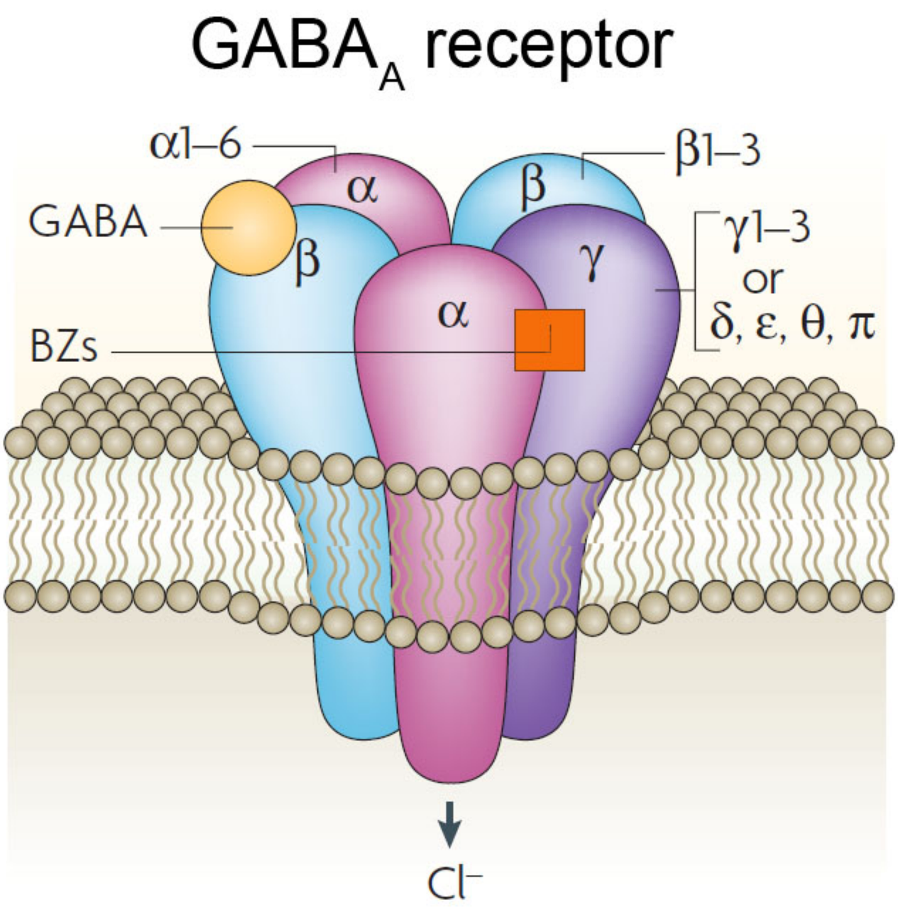

Figure 5. Schematic representation of the three-dimensional structure of the $GABA_A$ receptor. Five receptor subunits (2α, 2β, 1γ) are indicated immersed in the lipid bilayer of the cell membrane, delimiting a central ion channel permeable to the ion Cl^-. (figure taken from Jacob et al., 2008).

$GABA_A$ receptors formed by ρ subunit are located primarily in the retina and they are expressed on the cell membrane of the bipolar cells. They differ from $GABA_A$ and $GABA_B$ receptors as insensitive to both bicuculline and baclofen, but they are very sensitive to blockade of picrotoxin similar to $GABA_A$ receptors. They are formed by five ρ subunits and are therefore called homomeric receptors (Bormann, 2000).

1.3.3 $GABA_B$ receptors

Another class of GABA receptors are $GABA_B$ receptors. These receptors are heterodimers composed of two related subunits, $GABA_B$ R1 and $GABA_B$ R2 (Kaupmann et al., 1997; Bowery et al., 2002). The $GABA_B$ G-protein coupled receptors are activated selectively by GABA and his derivative, β-p-chlorophenyl GABA (baclofen) and, unlike $GABA_A$ receptors, are insensitive to bicuculline and muscimol. Other members of the G-protein coupled receptors are the metabotropic glutamate receptors, the muscarinic acetylcholine receptor and receptors for the dopamine, norepinephrine, histamine, and serotonin. They are protein complexes that span the cell membrane. Receptors coupled to a second messenger consist of three parts: the extracellular part, where glycosylation occurs; the transmembrane part, which forms a pocket where the neurotransmitter is presumed to act; and the intracytoplasmic part, where G-protein binding occurs. Occupation of such receptors alters the level of second messenger molecules that in turn affect among other targets ion channels (**Figure 6**).

The inhibitory effects of $GABA_B$ receptors on neuronal activity are mediated by decreased neurotransmitter release via inhibition of N/P/Q-type Ca^{2+} channels and by postsynaptic hyperpolarisation via the activation of GIRK channels (also known as inwardly rectifying K+ Kir3 channels) by Gβγ dimer (Mott and Lewis, 1994; Takahashi et al., 1998; Couve et al., 2000). So the main function of the receptors $GABA_B$ at presynaptic level is that of self-regulate the release of GABA when concentrations become excessively high in the synaptic cleft.

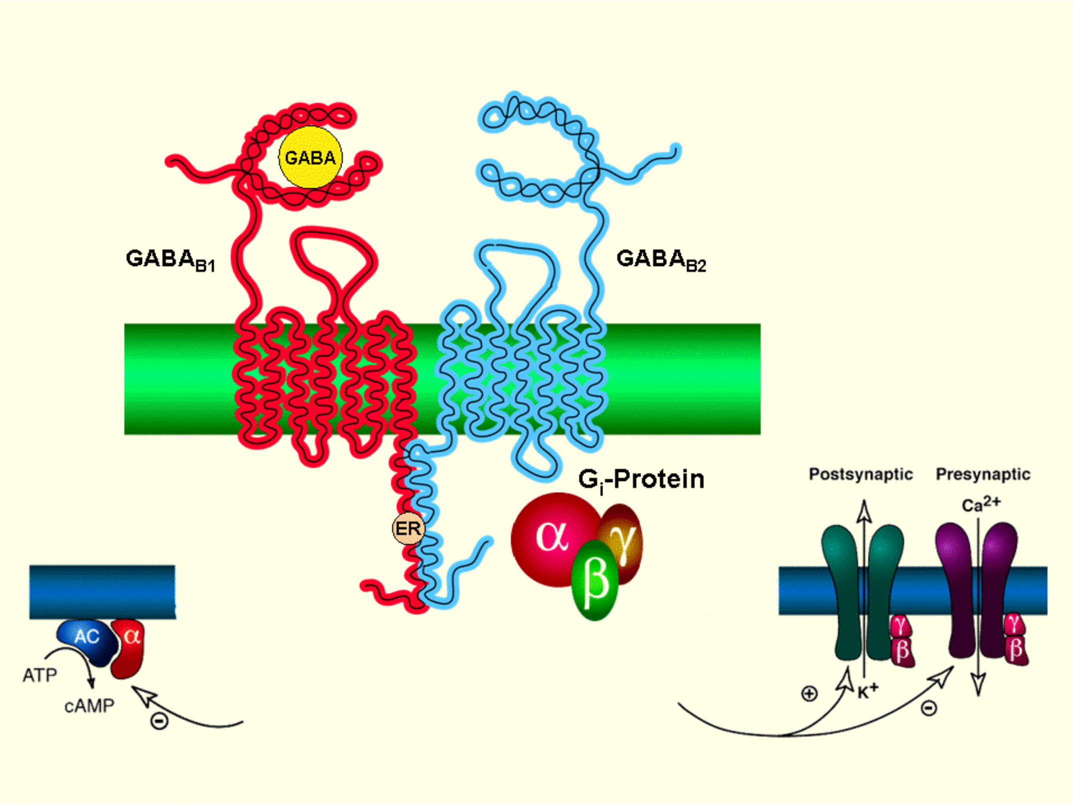

Figure 6. Structure of the GABA$_B$ receptor. (figure taken from University of Zurich, 2010 Impressum)

Because postsynaptic GABA$_B$ receptors are located at extrasynaptic sites away from GABA release sites, their activation is limited by GABA uptake and requires patterns of presynaptic activity that lead to GABA spillover and elevations of ambient GABA (Scanziani, 2000; Kulik et al., 2003). Under conditions of increased ambient GABA, such as occur with ischemia, epileptic seizures, or drugs that increase GABA concentration, coactivation of GABA$_A$ receptors and postsynaptic GABA$_B$ receptors will occur (Scanziani et al.,1991; During and Spencer, 1993; Wu et al., 2003). Postsynaptic GABA$_B$ receptors open G protein-activated inwardly rectifying potassium channels (GIRKs), which inhibit neuronal activity by local shunting and generate slow (100–500 ms) inhibitory postsynaptic potentials (IPSPs) that hyperpolarize the membrane (Gassmann and Bettler, 2012).

1.3.4 GABA$_A$ subunit composition

Initially, two subunits of a GABA$_A$ receptor have been purified from bovine brain using affinity chromatography (α, β) (Sigel et al., 1983; Sigel and Barnard, 1984). Subsequently the cDNAs coding for these subunits have been cloned (Schofield et al., 1987). Application of molecular biology techniques has allowed the cloning of different types of subunits ($\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}3}$, θ, ε, δ, π and $\rho_{1\text{-}3}$) (Barnard et al., 1998; Whiting, 1999) (**Figure 7**).

The purification of the receptor protein also provided the opportunity to raise monoclonal antibodies to the receptor, which were used to study the fine anatomical detail of receptor distribution.

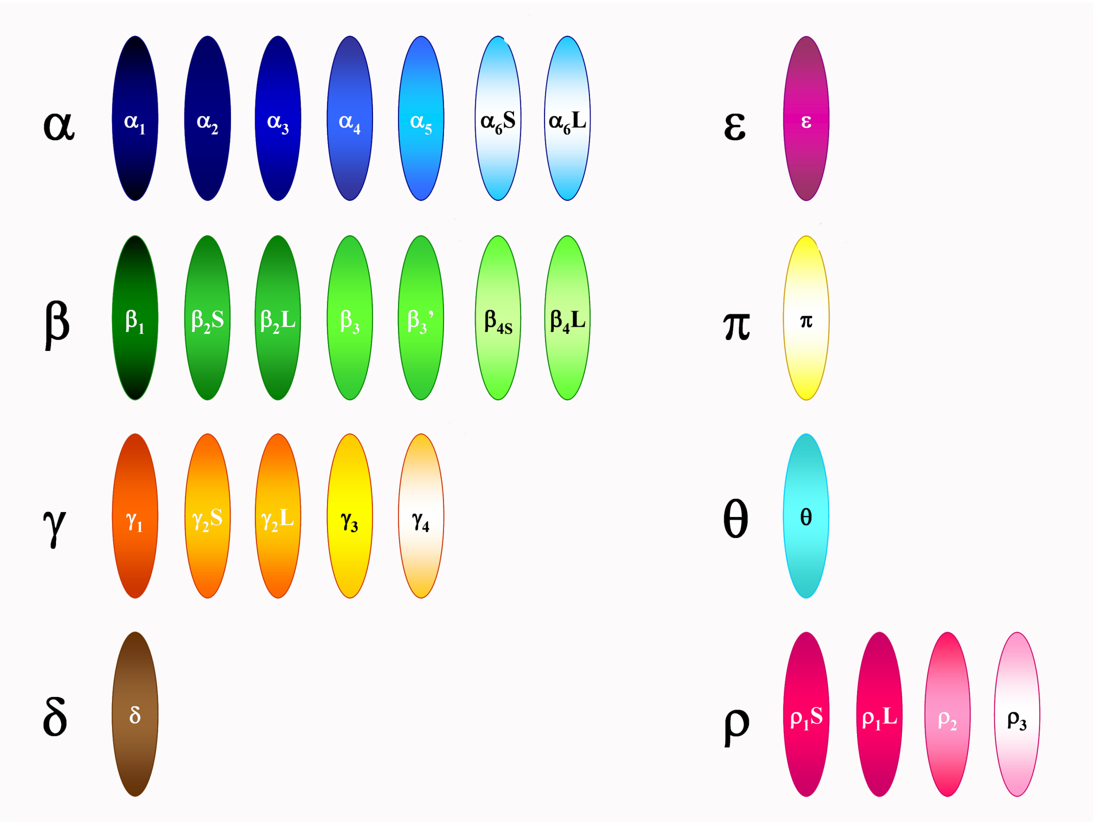

Figure 7. GABA$_A$ receptor subunits.

The $GABA_A$ receptor subunits are up to 450 amino acids residues in length and each of these has an amino terminal extracellular domain, four hydrophobic trans membrane domains (TM1 - TM4) forming a α helices (Schofield et al., 1987; Olsen and Tobin, 1990; Macdonald and Olsen, 1994; Hervers and Luddens, 1998), and a long intracellular loop between TM3 and TM4 containing specific sites for phosphorylation by Ser/Thr and Tyr dependent kinases (Mehta and Ticku, 1999) (**Figure 8**). Binding sites for the agonist GABA and for modulators of the benzodiazepine type are located in the N-terminal extra-cellular domain.

Based on the analogy to the nAChR, it is suggested that the N-terminal extra-cellular domain is built up by a 10-fold β-strand containing one α-helix. The transmem-brane helix M2 of each subunit contributes to the central pore, while helices M1, M3 and M4 form an outer shell to shield M2 from the lipids (Unwin, 2005). Recent crystalli-zation of a human homomeric β3 receptors has confirmed this picture (Miller and Aricescu, 2014).

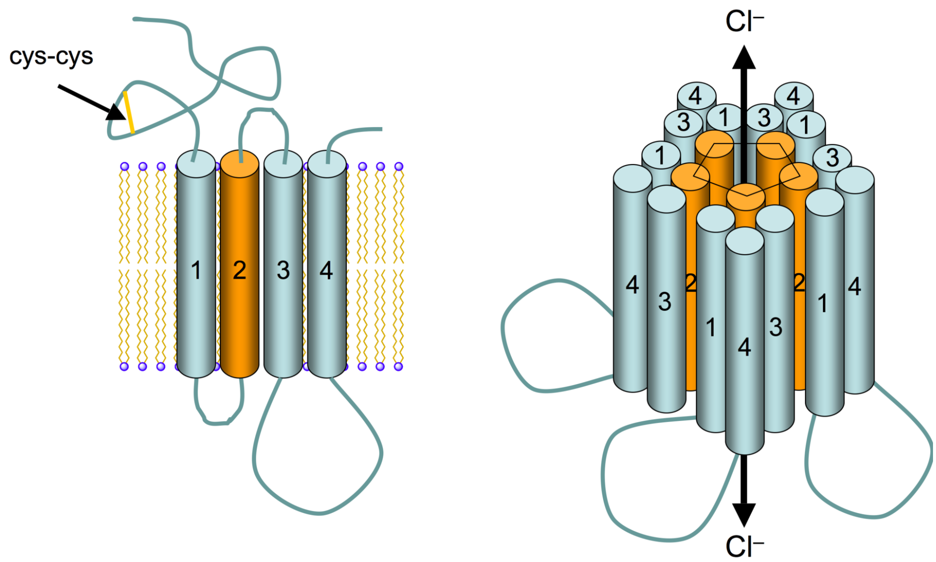

Figure 8. Topology of a subunit and the assembly of subunits. (figure taken from http://en.wikipedia.org/wiki/GABAA_receptor).

The different subunits have about 30-40% homology in their amino acid sequence and approximately 70% sequence homology between the subunit isoforms within a family (**Figure 9**) (Barnard et al., 1998; Whiting et al., 1999; Bonnert et al., 1999; Moragues et al., 2000; Bateson, 2004), and they are believed to be derived from a single common ancestral gene (Olsen and Tobin, 1990; Schofield et al., 1990; Seeburg et al., 1990; Burt and Kamatchi, 1991; Luddens and Wisden, 1991; Duggan et al., 1991; Doble and Martin, 1992; Wisden and Seeburg, 1992).

The different subunit isoforms are encoded by distinct genes localized on different chromosomes (Barnard et al., 1998; Whiting et al., 1999; Sieghart and Sperk, 2002).

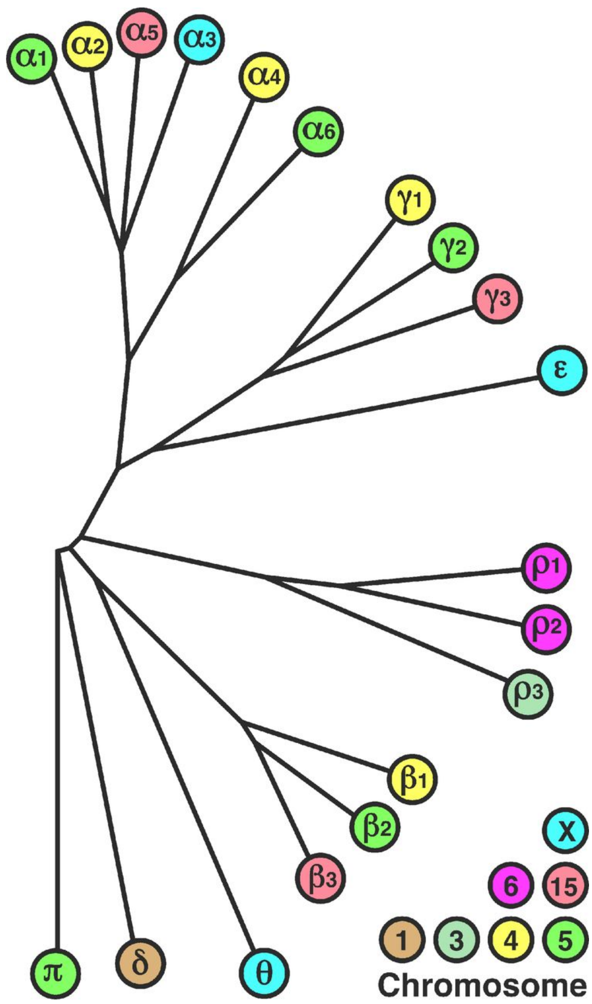

Figure 9. The subunits of the GABA$_A$ receptor.
The dendrogram is constructed based on the composition of the amino acid sequences of the different subunits of the GABA$_A$ receptor. The length of the segments that separate the different subunits is proportional to the evolutionary distance that separates their amino acid sequences. (figure taken from Sigel and Steinmann, 2012).

The diversity of receptor subunits is increased by alternative splicing, for example there are two forms of the γ_2 subunit, γ_2S (short) and γ_2L (long) (Kofuji et al., 1991; Whiting et al., 1990). These splice variants differ by the presence or absence of a short peptide in the intracellular loop between TM3 and TM4. Splice variants have also been detected for other subunits namely α_6, β_2, β_3, β_4 and ρ_1 (Sieghart and Sperk, 2002; Barnard et al., 1998). The subunits of the $GABA_A$ receptors share a high degree of homology with other subunits of the same receptor, the subunits of the nicotinic acetylcholine, the glycine and the serotonin (type 3) receptors. All these pentameric receptors share a near five-fold symmetry. The degree of symmetry is especially high in the second transmembrane domain M2 of these receptors that lines the ion channel. Amino acid residues of the α_1 $GABA_A$ receptor subunit, Ala^{253}, Val^{256}, Thr^{260}, Thr^{261} Leu^{263}, Thr^{264}, Thr^{267}, on the second transmembrane domain have been reported to be exposed to the channel lumen (Xu and Akabas, 1996).

The major receptor isoform in mammalian brain consists of α_1, β_2, and γ_2 subunits (Olsen and Sieghart, 2008). Concatenated $GABA_A$ receptor subunits studies have indicated a 2α:2β:1γ subunit stoichiometry for this receptor (Baumann et al., 2001; Baumann et al., 2002; Baur et al., 2006) with a subunit arrangement $\gamma\beta\alpha\beta\alpha$ anticlockwise as seen from the synaptic cleft (Baumann et al., 2001, Baumann et al., 2002; Baur et al., 2006). The presence of a defined subunit stoichiometry and arrangement in $\alpha\beta\gamma$ receptors indicates that assembly of $GABA_A$ receptors proceeds via defined pathways. The assembly of subunits seems to occur in the endoplasmic reticulum (ER) and to involve interaction with chaperone molecules (Connolly et al., 1996; Bollan et al., 2003 a,b). For the assembly of $GABA_A$ receptors, the chaperones calnexin, immunoglobulin heavy-chain-binding protein (BiP), and protein disulphide isomerase seem to be required.

The pharmacological properties depend both on subunit composition (Sigel et al., 1990) and arrangement (Minier and Sigel, 2004). It has been determined through immunoprecipitation experiments that in a single receptor complex two different α subunit isoforms can co-exist (Luddens et al, 1991). From the experimental point of view, a considerable number of receptor combinations have been reconstructed in vitro in different cell lines (Wong et al., 1992), although immunoprecipitation studies suggest that only a limited number of receptor subtypes is expressed in vivo in different neuronal populations (McKernan et al, 1996).

1.3.5 Synaptic and extrasynaptic $GABA_A$ receptors

Recently it has been shown that distinct subtypes of $GABA_A$ receptors are involved in two types of inhibitory control. The transient activation of synaptic $GABA_A$ receptors (due to the local release of GABA from the presynaptic terminal) is responsible of the classical "phasic" inhibition, while the persistent activation of $GABA_A$ of the extra synaptic receptors may generate a form of inhibition called "tonic" (**Figure 10**).

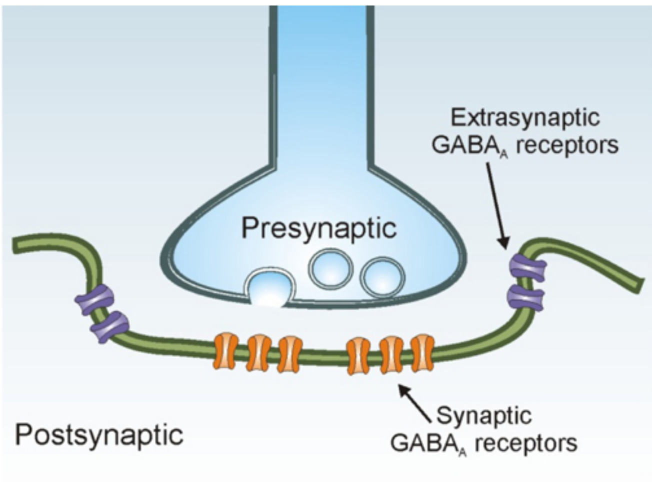

Figure 10. Synaptic and extra-synaptic receptors. The concentration of extracellular GABA is regulated by the release mechanisms, diffusion and uptake (recapture). GABA can escape from the synaptic cleft (spillover). (figure taken from Hunt et al., 2013).

The receptors that mediate tonic activity are continually exposed to GABA present in the extracellular environment ("spill over" of synapses and or released by astrocytes) (Nusser and Mody, 2002; Stell and Mody, 2002). These receptors have a very high affinity to GABA, and a slow desensitization, compared to receptors located in the synaptic space (Wallner et al., 2003). They are activated by very low concentrations of GABA (0.5-1 μM) (Mody, 2001) and for a very long time.

In contrast, the receptors in the synaptic space are activated by very high concentrations of GABA (≥ 1mM, close to saturation), directly released into the synaptic space and remain open for a very short period of time (≈ 10ms) (Wallner et al., 2003).

1.3.6 Pharmacological properties of $GABA_A$ receptors

The $GABA_A$ receptor is capable of mediating the action of several classes of compounds, many of which are of therapeutic importance. $GABA_A$ receptors constitute a selective target for numerous classes of CNS active drugs, including anxiolytics, sedativo-hypnotics, general anesthetics, anticonvulsants and myo-relaxants. Benzodiazepines, steroids, barbiturates and general anaesthetics are positive modulators, whereas β-carboline are negative modulators and picrotoxin, dieldrin are non-competitive channel blockers (**Figure 11**).

The positive modulators bind to allosteric sites on the receptor complex, causing increased efficiency of the GABA binding site and therefore an increase in Cl^- conductance; whereas the negative modulator have an opposed action, they produce an allosterically unfavorable conformation for GABA binding and therefore decreasing Cl^- conductance (Haefely, 1984; Paredes and Agmo, 1992). The non-competitive channel blockers bind to or near the central pore of the receptor complex and block Cl^- conductance through the ion channel. In order for $GABA_A$ receptors to be sensitive to the action of benzodiazepines they need to contain an α and a γ subunit, between which the benzodiazepine binds (Sigel et al., 2002; Sigel et al., 1998; Sigel and Buhr, 1997).

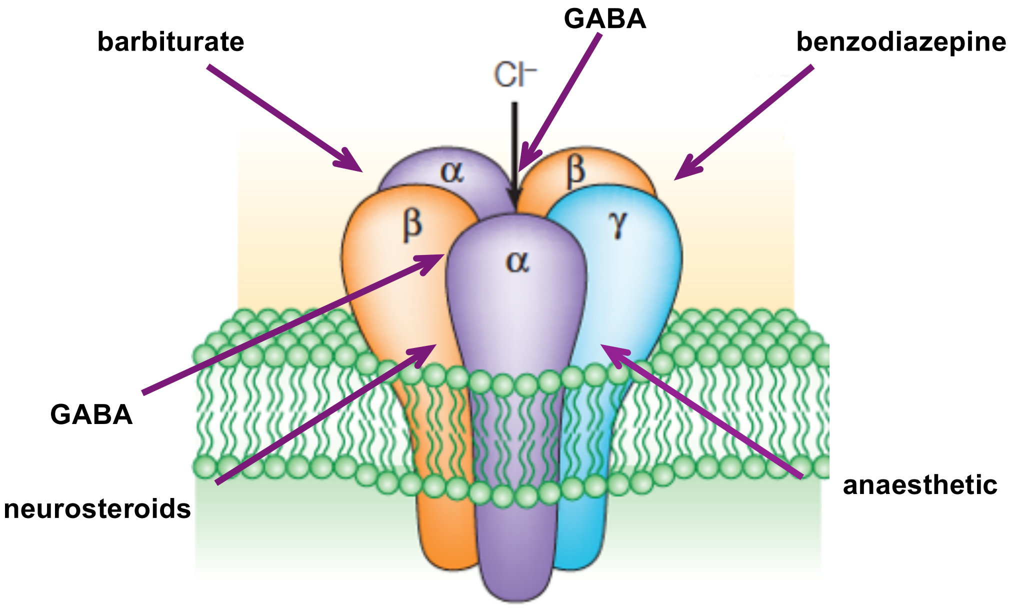

Figure 11. Pharmacology of the $GABA_A$ receptor. Schematic of the hypothetical molecular structure of the most abundant $GABA_A$ receptor composed of α,β and γ subunits. On the structure of the receptor complex the recognition sites for different positive modulators and negative, as well as for the neurotransmitter are indicated. (figure adapted from Belelli et al., 2005).

Once bound, the benzodiazepine locks the $GABA_A$ receptor in a conformation where the neurotransmitter GABA has much higher affinity for the $GABA_A$ receptor, increasing the frequency of opening of the associated chloride ion channel and hyperpolarising the membrane. This potentiates the inhibitory effect of the available GABA, leading to sedative and anxiolytic effects.

1.3.7 Effect of subunit composition on pharmacological properties

As mentioned in a previous chapter, the pharmacology of the $GABA_A$ receptors is influenced by its subunit composition and distribution and the receptors mediate two fundamentally distinct forms of inhibitory transmission, which depend on their localisation, either postsynaptic or extrasynaptic. Importantly, these two major populations of $GABA_A$ receptors are molecularly distinct, with postsynaptic receptors containing mainly the α_1, α_2 and α_3 subunits, along with β subunit variants and the γ_2 subunit, and extra-synaptic receptors containing the α_4, α_5 and α_6 subunits, often along with the δ subunit. This observation implies that the mechanisms of subcellular targeting of $GABA_A$ receptor subtypes are subunit specific, and can vary between CNS regions and developmental stages.

Several approaches such as mutation, gene knockout and the inhibition of expression of $GABA_A$ receptor subunits by antisense oligodeoxynucleotides have been used to establish the role of various subunits and their receptor assemblies. With the use of transgenic mice was discovered that different α subtype subunits mediate distinct pharmacological actions of benzodiazepines (Rudolph et al., 2004): the α_1 subunit mediates the sedative-hypnotic (Rudolph et al., 1999), the α_2 subunit mediates the anxiolytic effect and in part that muscle relaxant (Löw et al., 2000). The use of a pseudo-pregnant rat model and in vitro studies showed that α_4 subunit seems to be important in mediating the GABAergic transmission in some physiological conditions, (Smith et al., 1998) or pharmacological (Follesa et al., 2000) associated with abrupt changes in the levels of neurosteroids.

Furthermore recombinant receptors containing the α_4 and α_6 subunit bind with high affinity the benzodiazepine antagonist flumazenil, but very weakly classical benzodiazepines such as diazepam (Wisden et al., 1991). It has been shown that a histidine at the position α_1H101 and homologous position in α_2, α_3 and α_5 is crucial for benzodiazepine action and α_4 and α_6 subunit isoforms carry an arginine in this position (Wieland et al. 1992, Dunn et al. 1999).

The location of the picrotoxin binding site is controversial. Picrotoxin is reported to interact with residue Val256 (Xu, Covey and Akabas, 1995) and act as an open channel blocker or may inhibit channel function allosterically. (Ramakrishnan and Hess 2005). In favour of the first hypothesis is the study which show that the mutation in the homologous residue in Drosophila receptors confers cyclodiene and picrotoxin resistance (ffrench-Constant et al., 1993). Moreover the crystal structure of the homo-pentameric Caenorhabditis elegans glutamate-gated chloride channel (GluCL), which belong to the same cis receptor family like the $GABA_A$ receptor, show that picrotoxin directly occludes the pore near its cytosolic base of the lumen of the channel, at the 2' Thr and -2' Pro side chains (Hibbs and Gouaux, 2011).

1.3.8 $GABA_A$ receptors in peripheral tissues

Gamma-aminobutyric acid (GABA) and its receptors are found in a wide range of peripheral tissues, including parts of the peripheral nervous system, neuroendocrine cells including pancreatic (Taniguchi et al., 1979; Yang et al., 1994), pituitary (Racagni et al., 1983), and adrenal cells (Parramon et al., 1995). Subunits of GABA receptors have been demonstrated in non-neural tissues such as, lungs, gut, bladder, vascular, and uterine smooth muscle (Mizuta et al., 2008; Amenta et al., 1988; Ferguson and Marchant, 1995; Fujiwara and Muramatsu, 1975; Napoleone et al., 1991) in the immune system (Bergeret et al., 1998; Tian et al., 1999; Alam et al., 2005; Dionisio et al., 2013), in heart (Matsuyama, 1999), uterus (Hedblom, 1997; Neelands and Macdonald, 1999), kidney (Sarang et al., 2001) and liver (Erlitzki et al., 2000; Sun et al., 2003).

In contrast to the CNS, the role of GABA in the peripheral nervous system is not well defined. It has been known since 1950s that GABA exists in peripheral tissues of rodents and humans. The general focus has been to localise and identify GABAergic functions pharmacologically. Selected tissue are discussed in the following.

In airway smooth muscle cells, $GABA_A$ α_4, α_5, β_3, and γ_2 subunit proteins assemble in a functional $GABA_A$ receptor which seems to be responsible for the muscle relaxation, in fact the hyperpolarization induced by GABA results in a reduced intracellular calcium, which will not be able to activate the Ca^{2+}/calmodulin-dependent activation of myosin light chain kinase, resulting in reduced phosphorylation of myosin light chain leading to a less muscle contraction (Mizuta et al., 2008). This study has a high impact for the therapy of diseases such as asthma and chronic obstructive lung disease.

There is also evidence for the expression of inhibitory ligand-gated chloride channels in cells of the immune system. $GABA_A$ receptors are expressed in the human lymphocytes where mediate the communication between excitable cells on immune cells (Tian et al., 1999). Through these $GABA_A$ receptors, GABA can inhibit T cell responses to antigen both in vitro and in vivo inhibiting antigen-specific T cell proliferation. This function was mimicked by the $GABA_A$ receptor agonist muscimol, blocked by $GABA_A$ receptor antagonists and a $GABA_A$ receptor Cl^- channel blocker (picrotoxin) and enhanced by pentobarbital (Tian et al., 1999). This finding is of pharmacological importance because modulation of $GABA_A$ receptors may provide new approaches to modulate T cell responses in inflammation and autoimmune disease.

Moreover in patients seriously ill, on intensive care, the anaesthetic drugs propofol or thiopental are mostly used to induce sedation (Fraser and Riker, 2007). The use of this anaesthetic drugs in intensive care patients seem to increase the probability to develop serious infections, where 50% of patients with severe sepsis usually die (Nadal et al., 1995; Stover et al., 1998). According to a recent study, the increase in the risk of infection after the administration of the anaesthetic drug is due to their direct action on the $GABA_A$ receptors expressed in the monocytes, which inhibit the monocytes physiological function. The activation of $GABA_A$ receptors in the monocytes is inhibited by picrotoxin, but is not enhanced by the positive modulator diazepam. The expression of α_4, β_2, γ_1 and δ $GABA_A$ receptor subunits was detected in the monocytes. These receptors are insensitive to benzodiazepines, thus it is suggested from the authors that benzodiazepine could be consider as an alternative drug to propofol and thiopental in patients developing severe sepsis (Wheeler et al., 2011).

The concentrations of GABA in the peripheral tissues are generally low, about 1% of that in brain, with the exception of the female genital tract (Del Rio and Caballero, 1980) and pancreatic islets (Michalik and Ereciuska, 1992). In pancreatic islets, the GABA concentration is comparable to that of the CNS (Garry et al., 1986) and it is now clear that the activation of GABA receptors in islet β-cells increases insulin release (Bansan et al., 2011) exerts protective and regenerative effects on islet β-cells and reduces apoptosis in cultured islets (Dong et al., 2006).

The role of the $GABA_A$ receptor in liver is described in more detail in chapter four of the thesis.

1.4 Aims

$GABA_A$ receptors bind and are inhibited by epileptogenic agents such as picrotoxin, and cyclodiene insecticides such as dieldrin. The interaction site for picrotoxin is controversial. It may bind in the channel lumen and act as an open channel blocker or bind in a different binding sites and act allosterically. The aim of this project was to describe a novel inhibitor of rat $GABA_A$ receptors, the pentacyanocyclopentdienyl anion ($PCCP^-$), an aromatic monovalent anion with five-fold symmetry. $GABA_A$ receptors show near five-fold symmetry that is most pronounced in the second transmembrane domain M2 lining the Cl^- ion channel. $PCCP^-$ inhibited currents elicited by GABA with comparable potency as picrotoxin. This inhibition showed all characteristics of an open channel blocker, characterized by an apparent desensitization of the current, an off-current and sensitivity to the membrane potential. Other anion selective cys-loop receptors were also inhibited by $PCCP^-$, e.g. the Drosophila RDL $GABA_A$ receptors, carrying instecticide resistence mutations. The thesis aimed to identify amino acid residues in M2 involved in the recognition of $PCCP^-$. We used the substitute-cysteine accessibility method where consecutive residues in putative channel-lining were mutated to cysteine one at the time. With electrophysiological experiments we investigated if $PCCP^-$ could protect the interaction between a positive charged cysteine reactive compound MTSET$^+$ with the engineered cysteine residues exposed in the channel.

An additional short term project in collaboration with an other PhD student Simon Middendorp of Prof. Sigel's laboratory was to characterised a light-switchable modulator of $GABA_A$ receptors called azopropofol (AP2). It was my responsabiity to investigate the switching kinetics of this compound in patch clamp experiments.

Another aim of the thesis was to investigate the role of $GABA_A$ receptors in the liver. The idea was based on an article (Minuk et al., 2007) showing expression of the $GABA_A$ receptor in the liver. We aimed to investigate expression pattern, localization and function of the hepatocytes $GABA_A$ receptors in model cell lines and healthy and cancerous hepatocytes. Due to its nature as chloride channel, which in other tissues regulate proliferation, we aimed to test the potential involvement in proliferation and liver regeneration.

2 Results

2.1 Manuscript 1: A pentasymmetric open channel blocker for Cys-loop receptor channels

Valentina Carta*, Michael Pangerl*, Roland Baur, Roshan Puthenkalam, Margot Ernst, Dirk Trauner, Erwin Sigel

* These authors contributed equally to this study

Abstract

γ-Aminobutyric acid type A receptors ($GABA_A$ receptors) are chloride ion channels composed of five subunits, mediating fast synaptic and tonic inhibition in the mammalian brain. These receptors show near five-fold symmetry that is most pronounced in the second trans-membrane domain M2 lining the Cl^- ion channel. To take advantage of this inherent symmetry, we screened a variety of aromatic anions with matched symmetry and found an inhibitor, pentacyanocyclopentdienyl anion ($PCCP^-$) that exhibited all characteristics of an open channel blocker. Inhibition was strongly dependent on the membrane potential. Through mutagenesis and covalent modification, we identified the region α_1V256-α_1T261 in the rat recombinant $GABA_A$ receptor to be important for $PCCP^-$ action. Introduction of positive charges into M2 increased the affinity for $PCCP^-$ while $PCCP^-$ prevented the access of a positively charged molecule into M2. Interestingly, other anion selective cys-loop receptors were also inhibited by $PCCP^-$, among them the Drosophila RDL $GABA_A$ receptor carrying an insecticide resistance mutation, suggesting that $PCCP^-$ could serve as an insecticide.

Keywords: GABA, $GABA_A$ receptor, channel block

Introduction

Symmetry pervades nature at all levels from nuclear physics to astronomy [1]. In biology, it enables complex functions to arise from a limited set of building blocks and associated genes. A case in point is protein assemblies, such as viral capsids or trans-membrane ion channels. The former often show icosahedral symmetry, allowing for the encapsulation of maximum space with a minimum number of protein components [2]. The latter are often multimeric, for instance tetrameric (voltage-gated potassium channels), pentameric (cys-loop receptors) or hexameric (Orai channels), with a central pore formed by membrane-spanning subunits. Following the establishment of a basic multimeric assembly early in evolution, a higher level of functional sophistication is sometimes achieved through subsequent desymmetrization, for instance through concatenation or heteromultimerization of closely related, yet distinct, subunits.

GABA$_A$ receptors are a particularly interesting class of pentameric ligand-gated ion channels. They are composed of five subunits surrounding a central chloride ion channel and represent the major inhibitory receptors in the mammalian central nervous system [3-6]. The most abundant receptor isoform in mammalian brain consists of α_1, β_2, and γ_2 subunits [7]. Various approaches have been used to derive the subunit stoichiometry for this receptor, which has been determined as $2\alpha{:}2\beta{:}1\gamma$ with a subunit arrangement $\gamma\beta\alpha\beta\alpha$ anti-clockwise as seen from the synaptic cleft [8-12]. The pharmacological properties depend on subunit composition [13] and arrangement [14]. The subunits of GABA$_A$ receptors share a high degree of homology with other subunits of the same receptors, as well as subunits of other Cys-loop receptors. All these receptors have a near five-fold symmetry. The degree of symmetry is especially high in the second trans-membrane domain M2 that lines the ion channel (**Figure 12A**).

GABA$_A$ receptors have a rich pharmacology and are targeted by numerous agents such as muscimol, picrotoxin, benzodiazepines and insecticides [15]. None of these ligands, however, takes advantage of the five-fold (or near five-fold) symmetry of the receptors and the availability of multiple, i.e. up to five, related contact sites. Encouraged by recent work on polyvalent ligands [16], we hypothesized that small symmetric or nearly pentasymmetric anions would serve as symmetry-adapted blockers of the anion-selective GABA$_A$ receptors.

A

α1	ESVP**A**RT**V**FGV**TT**V**LT**MT**T**LS
γ2	DAVPARTSLGITTVLTMTTLS
β2	DASAARVALGITTVLTMTTIS
α1	ESVP**A**RT**V**FGV**TT**V**LT**MT**T**LS
β2	DASAARVALGITTVLTMTTIS

B

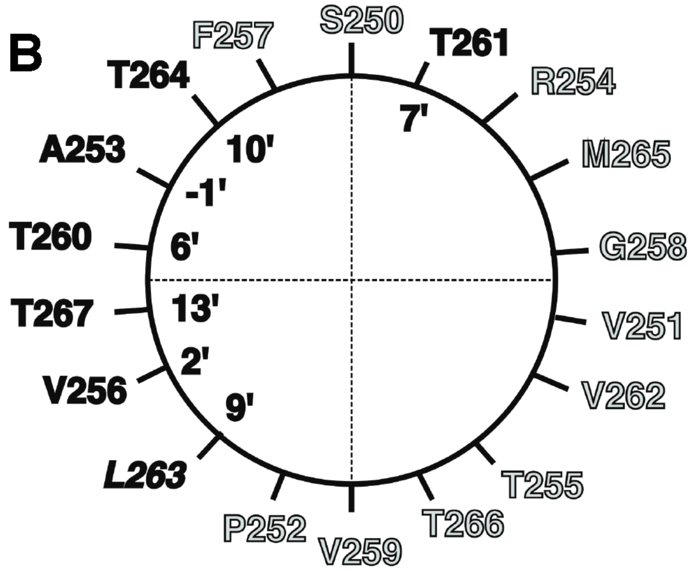

Figure 12. Aligned sequences of the amino acid residues in the subunits $\alpha_1\beta_2\gamma_2$ of the rat $GABA_A$ receptor. A, Alignment of 2α, 2β and 1γ subunit contributing to the formation of a $GABA_A$ pentamer. The residues in the α_1 subunit of the $GABA_A$ mutated to Cys are shown in boldface letters. **B**, α-Helical wheel representation of the rat α_1 M2 membrane-spanning domain showing the mutated residues in boldface letters.

Such molecules would have multiple similar interactions with the protein, which would result in a sharp increase of overall binding affinity (avidity) due to the polyvalency effect [17]. To test this hypothesis, we synthesized a range of perfectly or nearly five-fold symmetric anions (**Figure 13A**) and investigated them in electrophysiological experiments. Among these, we identified the pentacyanocyclopentdienyl anion ($PCCP^-$) as an inhibitor of $GABA_A$ receptors. Here we describe that $PCCP^-$ has all the hallmarks of an open channel blocker, discuss its binding site, and evaluate its interactions with other pentameric ligand-gated on channels.

Materials and Methods

Compounds 1 (Na^+PCCP^-) and 2 were synthesized using established literature protocols. Compounds 3 and 4 were synthesized from 2 by treatment with ammonia and hydrazine, respectively. Details of these syntheses will be published elsewhere.

Crystallographic data (excluding structure factors) for Na^+PCCP^- (acetone solvate) have been deposited with the Cambridge Crystallographic Data Centre as publication no. CCDC-946841. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge.

$MTSET^+$ was obtained from Toronto Research Chemicals Inc. All the other chemicals were purchased from Sigma-Aldrich.

The recombinant rat mutant subunits α_1A253C, α_1V256C, α_1T260C, α_1T261C, α_1L263C, α_1T264C, α_1T267C putatively facing the channel lumen (Fig. 1A,B) were prepared using the QuikChange mutagenesis kit (Stratagene).

Capped cRNAs were synthesized from the linearized plasmids. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase. The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain for visualization of the RNA. Known concentrations of RNA ladder were loaded as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19:1, the dried pellet dissolved in water and stored at -80°C. Xenopus oocytes were prepared, injected and defollicated as described previously [18] (Research approved by the Kantonstierarzt, Kantonaler Veterinärdienst Bern (Animal research permit BE98/12)). Briefly, Xenopus laevis oocytes were injected with 50nL of the cRNA solution containing wild type or mutated α_1, β_2 and γ_2 subunits at a concentration of 10 nM: 10 nM : 50 nM and then incubated in modified Barth's solution at 18°C for at least 24 h before the measurements. Homomeric glycine receptors (β-subunit), heteromeric glycine receptors (α and β-subunit) (cDNAs are a kind gift by B. Laube and H. Betz), the prokaryotic

ELIC (cDNA is a kind gift by R. Dutzler), the wild-type and the dieldrin resistant (RDL) mutant Drosophila $GABA_A$ receptor (wild type, bd splice variant and mutant A301S) (cDNAs are a kind gift by D. Sattelle) were also expressed.

Currents were measured using a home-built two-electrode voltage clamp amplifier in combination with a XY-recorder or digitized using a PowerLab 2/20 (AD Instruments) using the computer program Chart. Tests with a model oocyte were performed to ensure linearity in the larger current range. The response was linear up to 15 μA. Electrophysiological experiments were performed by using the two-electrode voltage clamp method at a holding potential of -80 mV. The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM Na-HEPES (pH 7.4) and was applied by a gravity flow of 6 ml/min. Wild type and and mutant receptors were characterized for their apparent affinity for γ-aminobutyric acid (GABA) for channel gating and for inhibition by $PCCP^-$ and picrotoxin. The GABA concentration response curve was determined by sequential application of increasing concentrations of GABA. Concentration-inhibition curves were performed at GABA (EC_{10}) by sequential co-application of GABA and increasing concentrations of $PCCP^-$ or picrotoxin. Inhibition was determined at the end of 1min co-application of GABA and $PCCP^-$ or picrotoxin. Concentration response curves were fitted with $I(c)= I_{max} / (1+(c/EC_{50})^n$, where I is the current potentiation, c is the concentration of GABA, I_{max} is the maximal current amplitude, EC_{50} is the concentration of GABA at which a half-maximal current amplitude was observed and n is the Hill coefficient. Concentration inhibition curves were fitted with $I(c) = I_{max} / (1+ (IC_{50}/c))$, where I is the control current amplitude, c is the concentration of $PCCP^-$ or picrotoxin, I_{max} is the control current amplitude elicited by GABA and IC_{50} is the concentration of $PCCP^-$ or picrotoxin at which half-maximal inhibition was observed. Drugs were applied as follows: 1 min GABA (EC_{10}), 1 min GABA (EC_{10}), 1 min GABA (EC_{10}) + $PCCP^-$ (IC_{50}), 1 min $MTSET^+$ (5 mM) either in the presence or absence of 100 μM GABA, 1 min GABA (EC_{10}), 1 min GABA (EC_{10}), 1 min GABA (EC_{10}) + $PCCP^-$ (concentration as before). Similar experiments were performed with picrotoxin.

Due to the difficulty of washing out picrotoxin out from the oocytes two different oocytes were used to test the inhibition before and after the treatment with the cysteine reactive compound. Inhibition after treatment was divided by % inhibition before treatment. To test the ability of $PCCP^-$ to protect the engineered cysteines from covalent modification by $MTSET^+$, we used the same sequence of perfusion except that $MTSET^+$ was co-applied with 1 mM $PCCP^-$. Results were obtained on 3-4 single oocytes for each receptor.The homology model is based on PDB entry 3RIF and was constructed with Modeller [19]. Ligand docking was performed with the GOLD software [20]. The binding site was defined to contain the 2´and 6' residues, side chains α_1T260, β_2T256 and γ_2T271 were kept flexible during docking.

Results

Synthesis and preliminary biological evaluation.

Figure 13A shows the investigated symmetry-adapted anions. $PCCP^-$ as its sodium salt 1 and compound 2 were synthesized following established literature procedures [21-23]. Compounds 3 and 4 were prepared from 2 by treatment with ammonia and hydrazine, respectively. Details of these syntheses will be published elsewhere. Compounds 1-4 were tested for inhibition of recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. Among these, only 1 was found to be highly active and was further characterized and the x-ray structure determined (**Figure 13B**).

Low concentrations of $PCCP^-$ inhibit currents mediated by $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors.

To evaluate $PCCP^-$ as an ion channel blocker, $\alpha_1\beta_2\gamma_2$ rat $GABA_A$ receptors were expressed in Xenopus oocytes. At concentrations of 0.3 - 10 μM $PCCP^-$ stimulated currents elicited by low concentrations of GABA (EC_1). This stimulation was highly variable between individual oocytes and was not mediated by the site for benzodiazepines as 1 µM Ro15-1788 fails to affect the stimulation. As stimulation was only observed at low concentration of GABA, we are tempted to assume a different site of action of $PCCP^-$ for stimulation and inhibition. At higher concentrations (> 1 µM), $PCCP^-$ induced an open-channel block, characterized by an apparent desensitization of the current and an off-current. As expected, this block became more prominent with increasing agonist concentrations. Stimulation became less evident. Original current traces and averaged data are shown in **Figure 14A,B**.

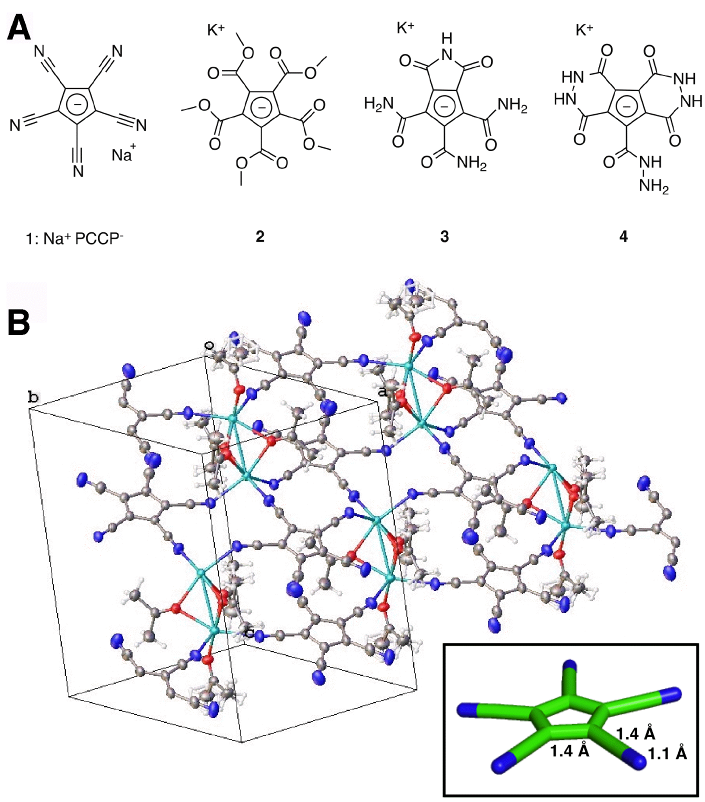

Figure 13. Symmetry-adapted anions, the chemical structure of $PCCP^-$ and the X-ray structure of Na^+PCCP^-. A, Symmetry-adapted anions. **B**, X-ray structure of Na^+PCCP^- (as the acetone solvate). The network of coordinative interactions between the partially negatively charged nitrogen atoms of $PCCP^-$ and the Na^+ cations is high-lighted. The insert indicates the geometry of the molecule.

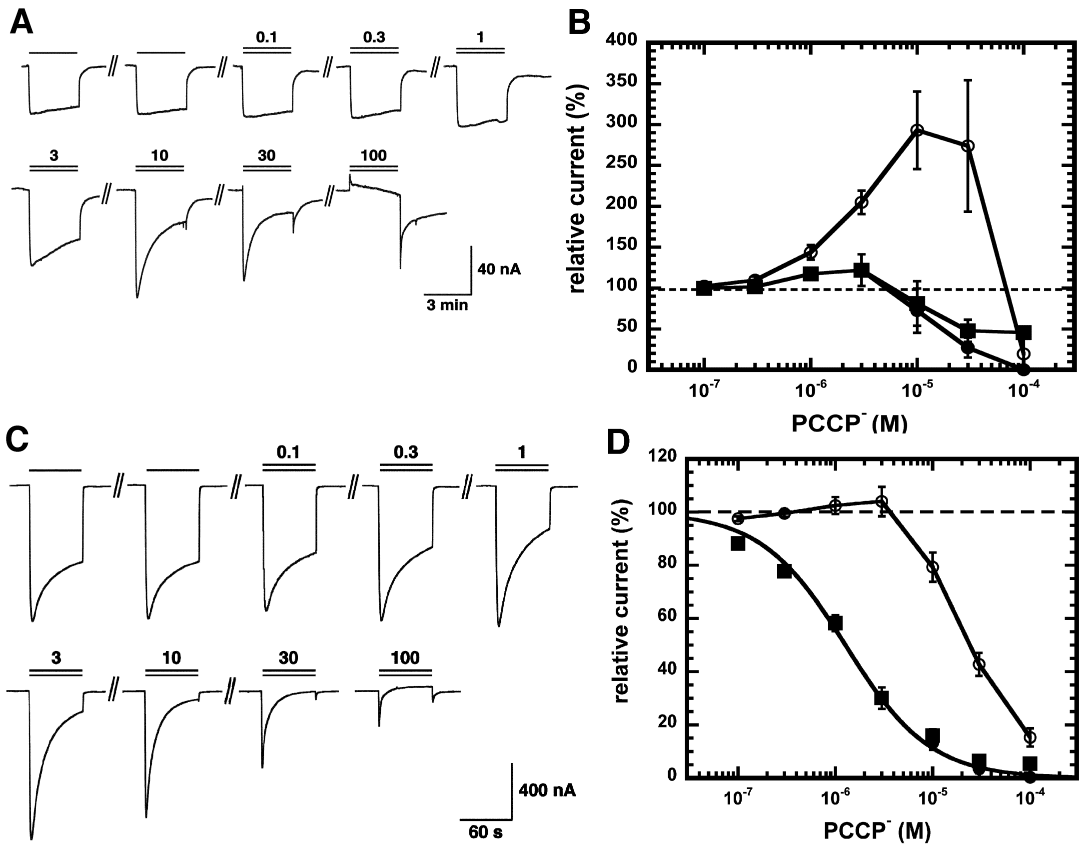

Figure 14. Effect of PCCP⁻ on recombinant $\alpha_1\beta_2\gamma_2$ GABA$_A$ receptors. A, GABA$_A$ receptors were expressed in Xenopus oocytes. The electrical currents recorded by two-electrode voltage clamp were activated with a concentration of GABA eliciting 1% of the maximal current amplitude (EC_1) and inhibited with increasing concentrations of PCCP⁻. The lower bar indicates the time of GABA application, the upper bar the time of PCCP⁻ application. The numbers indicate the concentration of PCCP⁻ in μM. At concentrations > 1 μM, induces an open-channel block, characterized by an apparent desensitization of the current and an off-current. **B**, Averaged concentration inhibition curve by PCCP⁻. Individual curves were fitted and standardized to the current elicited by GABA. Data are shown as mean ± SEM (n = 4). Open circle: peak current amplitudes at the beginning of the drug application. Filled squares: current amplitudes at the end of the drug application. Filled circles: current amplitudes at the end of the drug application corrected for the direct effect of PCCP⁻ on membranes. **C**) and **D**) same experiment carried out at a concentration of GABA eliciting 10% of the maximal current amplitude (EC_{10}).

Figure 14C,D shows a similar experiment carried out at a higher GABA concentration (EC_{10}). At this GABA concentration, $PCCP^-$ only exhibited a channel block. Current amplitudes measured after 1 min application of GABA and $PCCP^-$ were fitted with an IC_{50} of 2.6 ± 0.8 μM (n = 4). In additional experiments $PCCP^-$ was pre-applied for 30 s before the combined application of $PCCP^-$ with GABA. Current traces looked the same as without pre-application, indicating that $PCCP^-$ did not interact with closed channels.

$PCCP^-$ also inhibited $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\delta$ rat $GABA_A$ receptors, respectively, with an IC_{50} of 12.5 ± 4.8 μM (n = 4) and 0.71 ± 0.28 μM (n = 4) (**Figure 15 A**). It should be noted that the primary sequences of β_1 and β_2 differ substantially in the inner leaflet of M3.

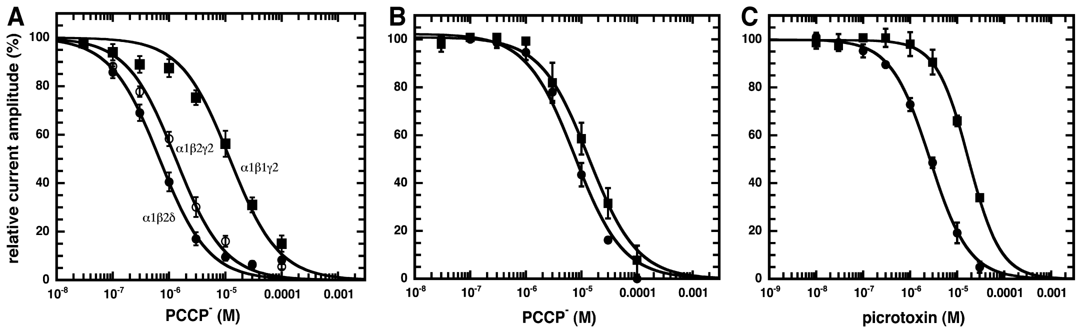

Figure 15. Concentration inhibition curves for rat and Drosophila $GABA_A$ receptors. A, Concentration inhibition curve for $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\delta$ $GABA_A$ receptors. Increasing concentrations of $PCCP^-$ were applied together with GABA EC_{10}. Individual inhibition curves were standardized and subsequently averaged (Mean ± SD, n=4). Data are compared with those obtained from $\alpha_1\beta_2\gamma_2$ receptors. Inhibition of the wild type and mutant RDL Drosophila $GABA_A$ receptor by $PCCP^-$ and Picrotoxin.

Concentration inhibition curves of (**B**) $PCCP^-$ and, (**C**) picrotoxin were determined in wild type (circles) and mutant (squares) receptors. Currents were activated with a concentration of GABA eliciting 10% of the maximal current amplitude (EC_{10}) and inhibited with increasing concentration of $PCCP^-$ or picrotoxin. Individual curves were standardized to initial current amplitudes and subsequently averaged. Data are shown as mean ± SD (n = 3).

Glycine homomeric and heteromeric receptors [24] were similarly inhibited, while ELIC [25] required about 100 μM $PCCP^-$ for half-maximal inhibition.

Inhibition by $PCCP^-$ was strongly dependent on the membrane potential (**Figure 16**). IC_{50} was 16.2 ± 1.3 μM (n = 3) at -120 mV, 6.3 ± 1.3 μM (n = 3) at -80 mV and 1.8 ± 0.8 μM (n = 3) at -40 mV. It should be noted that the IC_{50} at -80 mV was for unknown reasons somewhat higher than determined in the experiments before. From these values one can estimate the fraction of the voltage field experienced by the blocking particle at its blocking site from the equation derived by Woodhull [26] where δ is the fraction of the voltage field sensed by the blocker from the outside of the membrane.

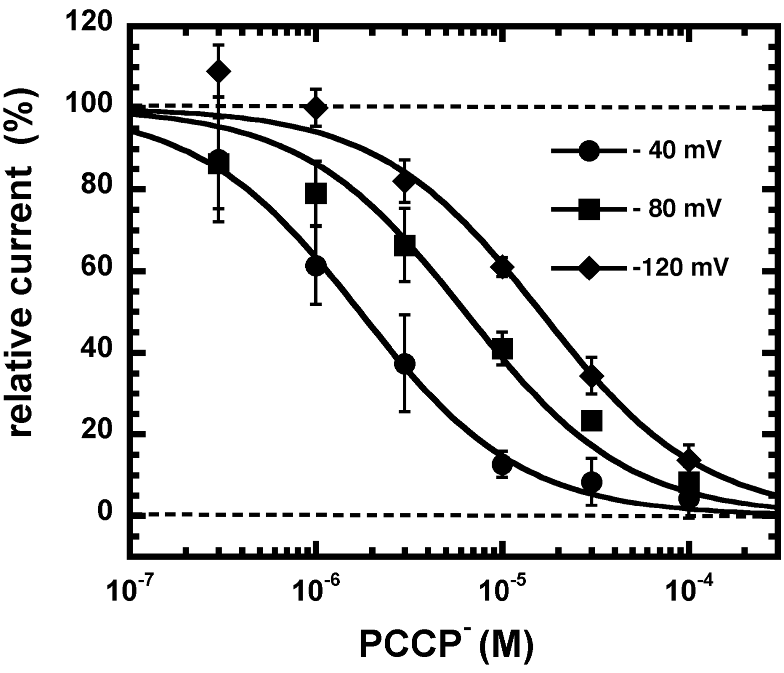

Figure 16. Effect of the membrane potential on inhibition by $PCCP^-$. A, $GABA_A$ receptors were activated with a concentration of GABA eliciting 10 % of the maximal current amplitude (EC_{10}) and inhibited with increasing concentrations of $PCCP^-$. Averaged concentration inhibition curves by $PCCP^-$ are shown for different membrane potentials. Individual curves were fitted and standardized to the current elicited by GABA. Data are shown as mean ± SD (n = 3)

δ was estimated to be ~ 0.7 the distance of the voltage field from the extracellular side.

In additional experiments it was tested of inhibition by $PCCP^-$ was of competitive or non-competitive nature. GABA concentration response curves were carried out in the absence of $PCCP^-$ or the presence of 2 μM or 10 μM $PCCP^-$. Analysis was complicated by the fact that currents were determined after 1 min application of GABA, when a substantial proportion of the channels had been desensitized. Nevertheless data showed rather a leftward shift of the GABA concentration response curve with increasing concentrations of $PCCP^-$, a phenomenon excluding competitive inhibition.

High concentrations of $PCCP^-$ interact with the lipid bilayer.

At concentrations ≥ 10 μM, $PCCP^-$ induced a current in non-injected oocytes. Perfusion of 10, 30 or 100 μM $PCCP^-$ at a holding potential of -80 mV resulted in small outward currents (**Figure 17B**). Upon a voltage jump from -80 to -30 mV, μA sized currents of more than 30 ms duration were observed in the presence of 100 μM $PCCP^-$ (**Figure 17A**). Thus it appears that $PCCP^-$ is able to insert into the bilayer and diffuse through the bilayer, reflecting its lack of dipole moment and relative lipophilicity (clogP = -0.48). It should be noted that the concentrations of $PCCP^-$ required to induce currents in non-injected oocytes are much larger than the concentrations required to inhibit $GABA_A$ receptor channels.

$PCCP^-$ inhibits currents mediated by wild type and mutant RDL Drosophila $GABA_A$ receptor to a similar extent.

Mutation A302S in RDL Drosophila $GABA_A$ receptors has been reported to confer a certain degree of resistance to inhibition by picrotoxin [27,28]. In our hands, a 7-fold reduction in sensitivity to picrotoxin upon the mutation was observed. Interestingly, $PCCP^-$ showed a much smaller (about 2-fold) difference in the IC_{50} between wild type and mutant receptors (**Figure 15B,C**).

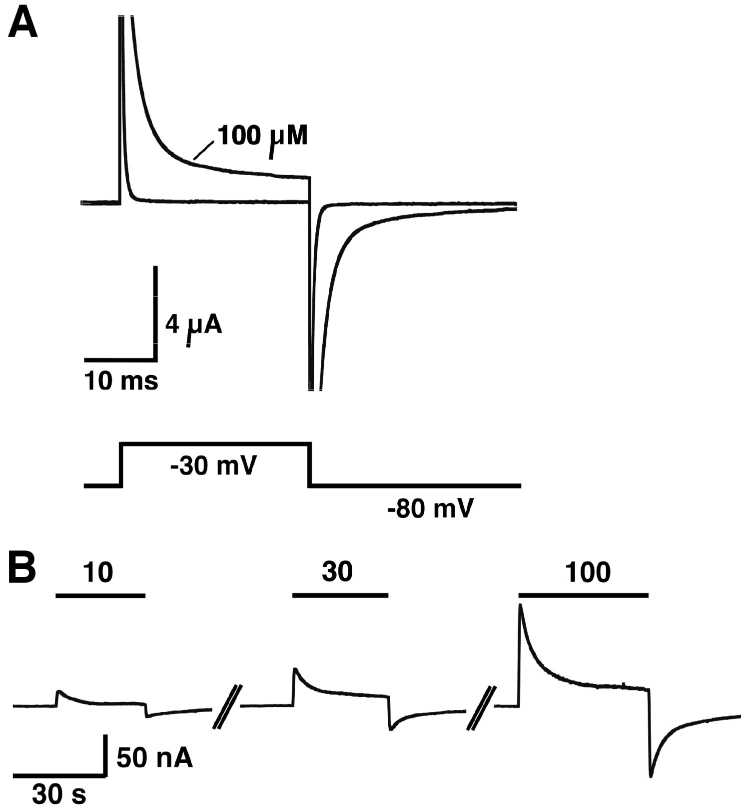

Figure 17. High concentration of PCCP$^-$ induce a current in non-injected Xenopus oocytes. A, Voltage jump of 25 ms duration from -80 mV to -30 mV. A μA sized transient current flows with each voltage step. **B**, A small transient outward current is induced after applications of 10, 30, and 100 μM PCCP$^-$ of 30 s duration to an oocyte held at a membrane potential of -80 mV

Pharmacological properties of mutant receptors.

To identify important residues for the interaction with PCCP$^-$, we mutated, one at a time, seven residues in M2 of α_1 to cysteine residues. (**Figure 12A,B**). Mutated α_1 subunits were co-expressed with wild type β_2 and γ_2 subunits in Xenopus oocytes. The sensitivity to GABA was tested for the different mutants. Expression of α_1L263C$\beta_2\gamma_2$ resulted in a spontaneously open channel. While α_1A253C$\beta_2\gamma_2$, α_1T260C$\beta_2\gamma_2$, α_1T267C$\beta_2\gamma_2$ showed an EC_{50} similar to wild type receptors, the mutant α_1V256C$\beta_2\gamma_2$ showed an about 10-fold increase and the mutants α_1T261C$\beta_2\gamma_2$ and α_1T264C$\beta_2\gamma_2$ showed an about 4 fold decrease in the EC_{50} for GABA (**Figure 18A; Table 1**). The sensitivity to inhibition by PCCP$^-$ of GABA-activated currents was also determined for wild type and mutant receptors. A GABA concentration eliciting 10% of the maximal current in the corresponding receptor was used in these experiments. The mutant receptors $\alpha_1V256C\beta_2\gamma_2$ and $\alpha_1T261C\beta_2\gamma_2$ displayed each an about 20-30 fold reduced sensitivity to PCCP$^-$ compared with wild type receptors (**Figure 18B; Table 1**). The sensitivity of GABA-activated currents to picrotoxin was also determined using the same conditions as for PCCP$^-$ (**Fig. 18C; Table 1**). Little effect of the studied mutations was observed on the IC_{50} for picrotoxin.

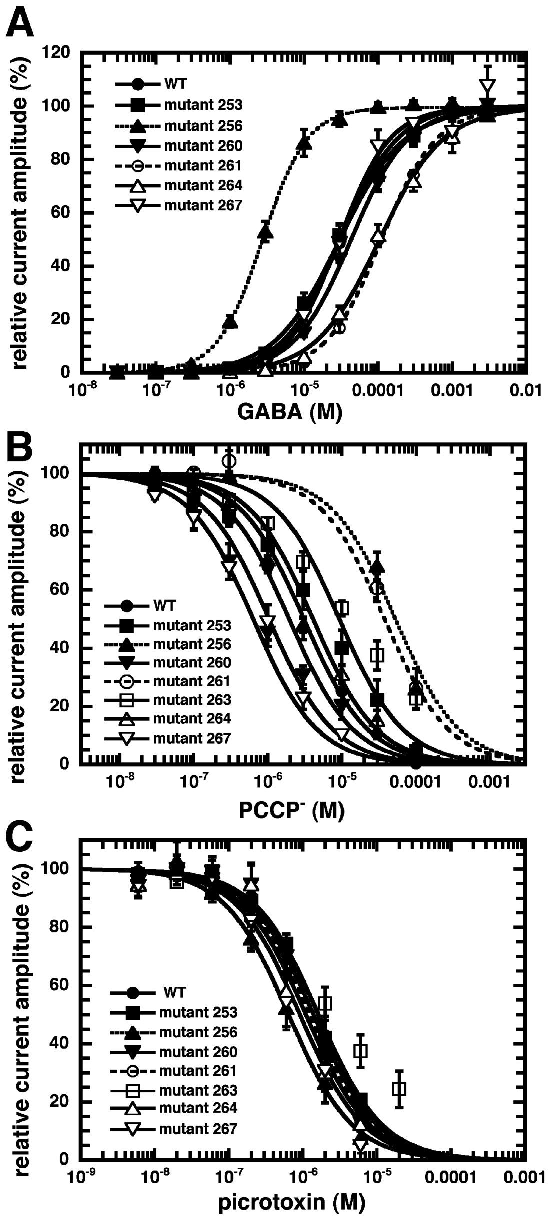

Figure 18. Pharmacological properties of wild type and cysteine mutant $GABA_A$ receptors. **A**, GABA concentration response curves from wild type and mutant rat $GABA_A$ receptor. **B**, PCCP$^-$ and **C**, picrotoxin concentration inhibition curves. Individual curves were fitted and standardized. Data are shown as mean ± SD (n=3 to 4)

Table 1. Pharmacological evaluation of the expressed recombinant receptors. EC_{50} for GABA, IC_{50} for $PCCP^-$, and IC_{50} for picrotoxin are given for wild type and mutant receptors.

Receptor	GABA EC_{50} (µM) mean ± SD	n	$PCCP^-$ IC_{50} (µM) mean ± SD	n	Picrotoxin IC_{50} (µM) mean ± SD	n
$\alpha_1\beta_2\gamma_2$	31.3 ± 6.5	3	2.58 ± 0.75	3	2.02 ± 0.89	6
α_1A253C$\beta_2\gamma_2$	30.6 ± 6.4	3	2.63 ± 0.31	3	1.79 ± 0.15	3
α_1V256C$\beta_2\gamma_2$	2.91 ± 7.4	4	62.2 ± 22.4	4	0.74 ± 0.36	4
α_1T260C$\beta_2\gamma_2$	44.1 ± 6.4	4	0.85 ± 0.46	4	1.31 ± 0.41	3
α_1T261C$\beta_2\gamma_2$	111 ± 1	3	46.4 ± 7.25	3	1.31 ± 0.71	3
α_1L263C$\beta_2\gamma_2$	open ch.	3	4.47 ± 1.36	3	1.10 ± 0.21	3
α_1T264C$\beta_2\gamma_2$	126 ± 24	4	3.15 ± 0.48	3	1.03 ± 0.35	3
α_1T267C$\beta_2\gamma_2$	32.2 ± 11.9	3	0.77 ± 0.11	3	0.83 ± 0.48	3

The effect of $MTSET^+$ on the cysteine-substitution mutants and the binding site of $PCCP^-$.

A cysteine scan was chosen as it allows covalent reaction with a cysteine reactive compound to potentially increase the effect seen with the mutation alone, to test accessibility of the residue and to investigate protection from the covalent reaction by a compound. In preliminary experiments $pCMBS^-$, $MTSEA^+$ and $MTSET^+$ were tested. Only treatment with $MTSET^+$ left wild type $GABA_A$ receptors unaffected (**Figure 19A**). Therefore $MTSET^+$ was chosen for further experimentation. Current traces for wild type receptor and α_1V256C$\beta_2\gamma_2$ and α_1T260C$\beta_2\gamma_2$ mutant receptors are shown in **Figure**

19B,C and **Figure 20A,B**, illustrating typical experiments where we chose an inhibitor concentration such as to inhibit about 50% of the late current response elicited by GABA (EC_{10}) in the corresponding receptor.

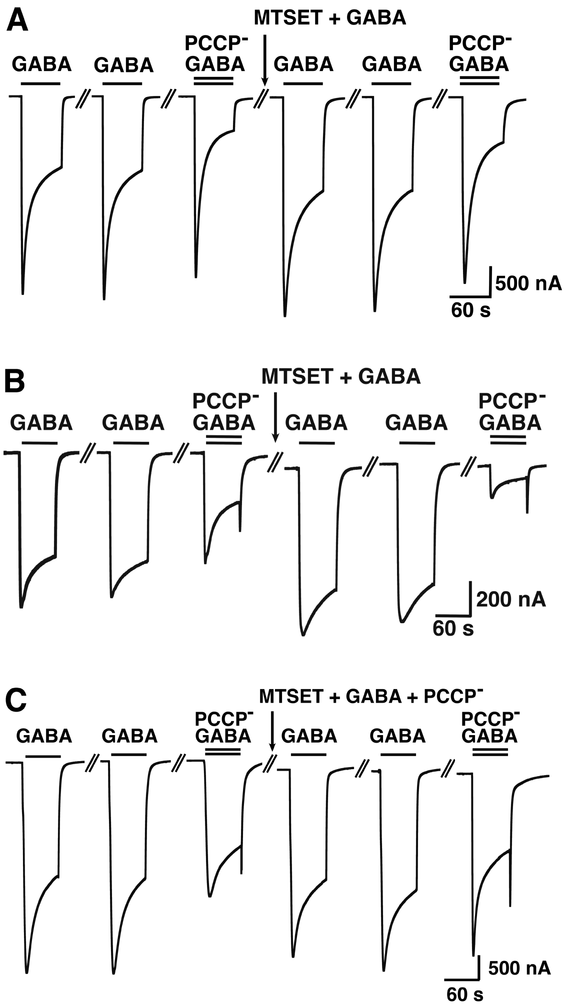

Figure 19. $PCCP^-$ prevents the increase in $PCCP^-$ sensitivity of α_1V256$\beta_2\gamma_2$ mediated by $MTSET^+$ + GABA. GABA (EC_{10}) was applied repetitively until a stable current response was observed followed by inhibition of the channel by $PCCP^-$. Subsequently 5 mM MTSET was applied in the presence of GABA. After $MTSET^+$ treatment GABA was applied twice followed by a combined application of GABA and the same concentration of $PCCP^-$ used before. **A**, Wild type receptors were not affected by this treatment. **B**, The treatment leads to an enhanced inhibition in α_1V256C$\beta_2\gamma_2$. **C**, 5 mM $MTSET^+$ was applied to α_1V256C mutant receptor in presence of GABA and 1 mM $PCCP^-$. $PCCP^-$ prevented enhanced inhibition and therefore covalent reaction. These experiments were repeated independently three times using different oocytes.

MTSET$^+$ + GABA treatment applied for 1 min to the wild type receptor had no effect on the affinity for PCCP$^-$ (**Figure 19A**). MTSET$^+$ + GABA treatment in α_1V256C$\beta_2\gamma_2$ led to an increase in PCCP$^-$ inhibition (**Figure 19B**). This effect could be prevented when 1 mM of PCCP$^-$ was present during the treatment (**Figure 19C**). The **Figure 20A** shows that MTSET$^+$ + GABA treatment leads to a spontaneously open channel in the α_1T260C$\beta_2\gamma_2$ mutant, characterized by a shift on the base line of the current. We applied increasing concentrations of PCCP$^-$ to investigate if PCCP$^-$ was able to block the channel again.

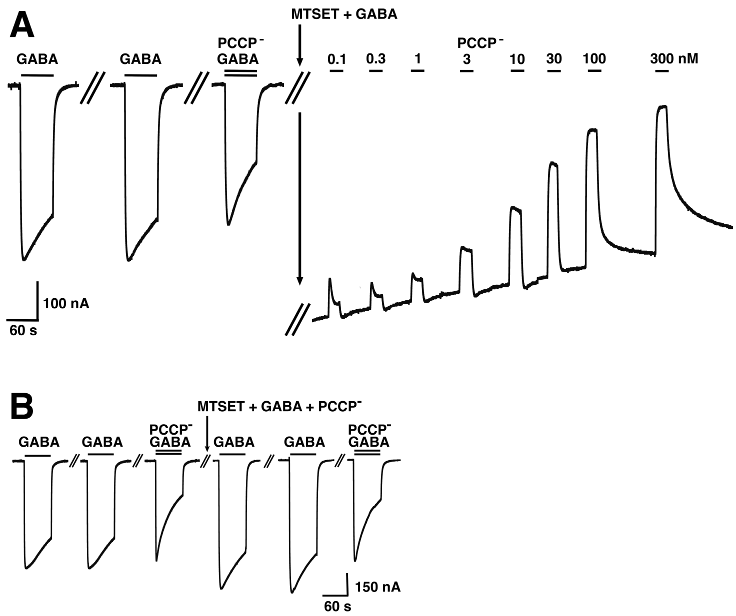

Figure 20 PCCP$^-$ prevents the increase in PCCP$^-$ sensitivity of α_1T260$\beta_2\gamma_2$ mediated by MTSET$^+$ + GABA. GABA (EC_{10}) was applied repetitively until a stable current response was observed followed by inhibition of the channel by PCCP$^-$. Subsequently 5 mM MTSET$^+$ was applied in the presence of GABA. After MTSET$^+$ treatment GABA was applied twice followed by a combined application of GABA and the same concentration of PCCP$^-$ used before. A, The treatment leads to a spontaneously open channel and an enhanced inhibition in α_1T260C$\beta_2\gamma_2$. B, 5 mM MTSET$^+$ was applied to α_1T260C mutant receptor in presence of GABA and 1mM PCCP$^-$. PCCP$^-$ prevents the open channel formation. These experiments were repeated independently three times using different oocytes.

The IC_{50} value after the treatment was 9 ± 0.3 nM (mean ± SD, n = 3) as compared to the IC_{50} of 0.85 ± 0.46 μM (n = 3) before the treatment. This indicates a strongly enhanced affinity for $PCCP^-$. Moreover, as the maximally inhibited current level almost reached the original base line we conclude that $PCCP^-$ was able to close the channel again. **Figure 20B** documents that presence of 1 mM $PCCP^-$ during $MTSET^+$ + GABA treatment prevents the open channel formation and the affinity for $PCCP^-$ was the same before and after the treatment.

Figure 21 summarizes our observations in wild type and mutant receptors. The bars indicate the ratio of the percentage of inhibition by $PCCP^-$ (or picrotoxin) observed after application divided by that before the application of $MTSET^+$ (black bars). The diagrams on the left show this ratio for $PCCP^-$ (**Figure 21A**) and picrotoxin (**Figure 21C**) after application of $MTSET^+$ without GABA and the diagrams on the right show the above ratio for $PCCP^-$ (**Figure 21B**) and picrotoxin (**Figure 21D**) after application of $MTSET^+$ in the presence of GABA. A value of 1 indicates that the IC_{50} before and after $MTSET^+$ treatment were the same. An increase in the value indicates a decrease in the IC_{50} of an inhibitor (increase in apparent affinity) and a decrease below 1 the opposite. $MTSET^+$ treatment in the absence of GABA caused generally little change in the apparent affinity of both channel inhibitors (**Figure 21A,C**). An exception is the mutant α_1L263C$\beta_2\gamma_2$ with a significant increase in the ratio for $PCCP^-$ ($p < 0.01$) and a significant decrease in the ratio for picrotoxin ($p < 0.001$). This mutation leads to an open channel, to the lumen of which $MTSET^+$ obviously has access in the absence of GABA. For those mutations already slightly affected by the $MTSET^+$ treatment alone, the effect on the inhibition by $PCCP^-$ increased when the $MTSET^+$ treatment was carried out in presence of 100 μM GABA. The application of $MTSET^+$ + GABA caused a significant increase in the % inhibition by $PCCP^-$ in α_1V256C$\beta_2\gamma_2$, α_1T260C$\beta_2\gamma_2$, α_1T263C$\beta_2\gamma_2$, α_1T264C$\beta_2\gamma_2$ and α_1T267C$\beta_2\gamma_2$ (each $p < 0.01$) mutants (**Figure 20B**).

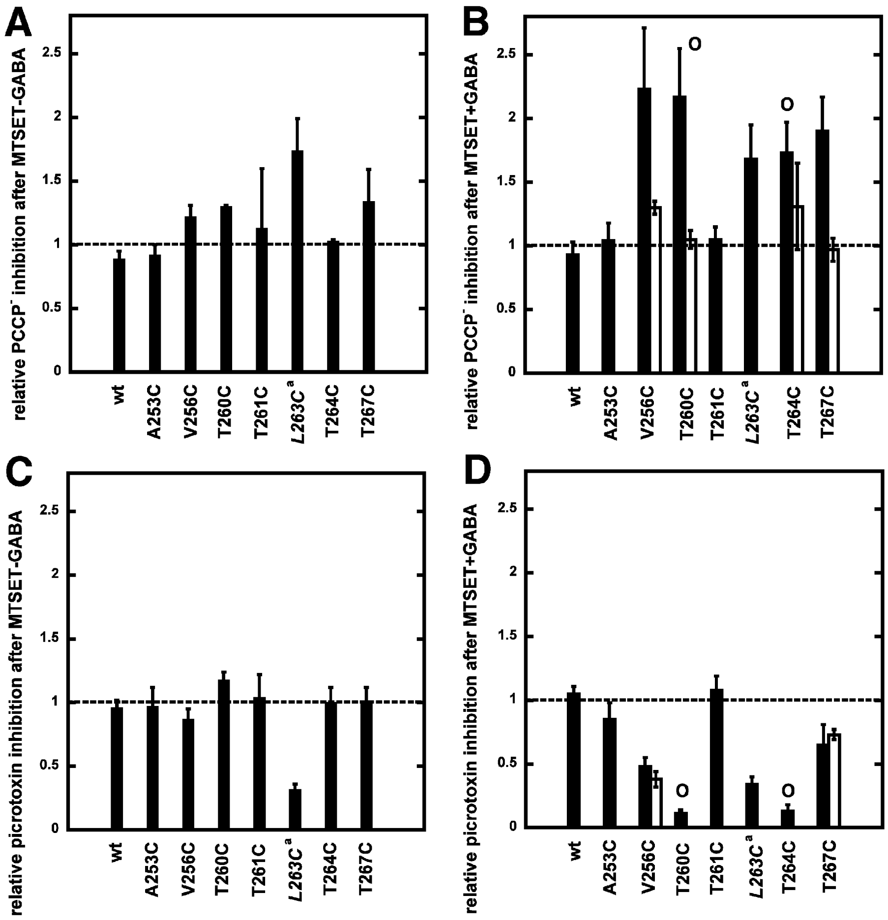

Figure 21. Effect of MTSET$^+$ on the PCCP$^-$ and picrotoxin inhibition of wild type and mutants GABA$_A$ receptors. Currents were elicited with GABA EC$_{10}$. The concentration of PCCP$^-$ or picrotoxin was chosen such as to inhibit about 50% of the late current response in the corresponding receptor. Subsequently oocytes were treated with the cysteine-reactive reagent MTSET$^+$ and inhibition by the same concentration of PCCP$^-$ or picrotoxin was determined. The ratio of the inhibition after treatment divided by inhibition before treatment is shown as a bar. **A, C,** MTSET$^+$ was applied in the absence of GABA. **B, D,** MTSET$^+$ was applied in the presence of 100 μM GABA. The circle symbol on top of the bar for the T260C and T264C mutations indicates formation of an open channel after MTSET$^+$ treatment when applied in the presence of GABA. The white bars show results of experiments where the MTSET$^+$ + GABA treatment was performed in the presence of 1 mM PCCP$^-$ or 1 mM picrotoxin, in order to see if the covalent reaction could be prevented by the channel blockers. The asterisks sign (*) indicates that 1 mM picrotoxin was not able to suppress formation of open channels. Mean ± SD is shown. The number of oocytes for each experimental condition is either three or six.

Discussion

Based on symmetry considerations, we have identified an aromatic monovalent anion with five-fold symmetry, $PCCP^-$, as inhibitor of rat $GABA_A$ receptors. The exposure to increasing concentrations of $PCCP^-$ causes inhibition in the GABA-evoked current typical for an open channel blocker. All the pentameric receptors belonging to the Cys-loop family share a near five-fold symmetry which is most pronounced in the second transmembrane domain M2. $PCCP^-$ can also inhibit glycine homomeric and heteromeric receptors with similar affinity to the $GABA_A$ receptor and with smaller affinity ELIC. Interestingly, $PCCP^-$ also inhibits Drosophila wild type and mutant RDL channels carrying the dieldrin resistance mutation [27,28] suggesting a possible use of $PCCP^-$ as insecticide. High concentrations of $PCCP^-$ induced currents by themselves. We ascribe this phenomenon to distribution of $PCCP^-$ into the lipid bilayer and permeation through the bilayer. $PCCP^-$ lacks a dipole moment and is therefore comparatively lipophilic with a clogP of -0.48. However, we can not exclude an action of $PCCP^-$ on channels endogenous to the oocyte.

We studied the molecular site of interaction of $PCCP^-$ on $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. A series of cysteine mutations were introduced in M2 into amino acid residues of the α_1 subunit. We selected residues that have been proposed to line the ion channel α_1A253, α_1V256, α_1T260, α_1T261, α_1L263, α_1T267 [29,30] (**Figure 12A,B**). The mutant receptors α_1L263C$\beta_2\gamma_2$ form an open channel that could not be activated by GABA. This leucine residue is conserved in all known subunits of acetylcholine, glycine and $GABA_A$ receptors. It has been postulated that this residue plays a role in the gating mechanism of the channel, where the closure is achieved when the large hydrophobic leucine residues move into the channel inhibiting ion flux (for review see [31]). Most of the mutations studied here had little effect on the apparent affinity to GABA. Mutations in residues α_1V256 and α_1T261 each caused an approximately 30-20 fold decrease in the apparent affinity of $PCCP^-$ to inhibit currents induced by GABA. This may suggest that $PCCP^-$ directly interacts with these residues, but it cannot be excluded here that these two residues allosterically affect the $PCCP^-$ binding site. The mutations did not affect the apparent affinity for picrotoxin to inhibit current elicited by GABA.

It was of interest to test accessibility of the introduced cysteines to a cysteine reactive reagent. $MTSET^+$ was chosen as it had a negligible effect on wild type receptors. It should be noted that a receptor pentamer has two α_1 subunits and that covalent reaction of a mutated receptor with $MTSET^+$ introduces two positive charges. Evidence was obtained that $MTSET^+$ has better access to the channel lumen in the presence of the channel agonist GABA and can penetrate as far as α_1V256. Similar observations have been made by Xu et al. [32] using a different cysteine reactive reagent.

Interestingly, covalent reaction of $MTSET^+$ with α_1V256C$\beta_2\gamma_2$, α_1T260C$\beta_2\gamma_2$, α_1L263C$\beta_2\gamma_2$, α_1T264C$\beta_2\gamma_2$ and α_1T267C$\beta_2\gamma_2$, led to an increase in the apparent affinity for $PCCP^-$ for channel inhibition. The fact that introduction of a relatively bulky moiety leads to an increase in affinity is probably due introduction of positive charges that favorably interact with the negatively charged $PCCP^-$. Covalent reaction of $MTSET^+$ with α_1V256C$\beta_2\gamma_2$, α_1T260C$\beta_2\gamma_2$ and α_1T267C$\beta_2\gamma_2$ was prevented in the presence of $PCCP^-$. The simplest interpretation of our observations including the direct effect of the mutations α_1V256C and α_1T261C on the affinity of $PCCP^-$ is that $PCCP^-$ can penetrate almost down to the level of α_1V256C. Binding of $PCCP^-$ then prevents $MTSET^+$ access by channel constriction (**Figure 22**). However, we cannot exclude that $PCCP^-$ has a second binding site at the level of α_1T267 that is not sensitive to the mutation of this residue to cysteine.

Hydrophobic anions have previously been described to inhibit $GABA_A$ receptors in a voltage independent fashion [33] reportedly in the absence of a conventional ligand binding site. Similar to the observations made here the mutation of residue α_1V256 located in M2 affected inhibition. We can not fully exclude the possibility of an action of $PCCP^-$ outside the channel, but following observations are in line with the existence of a binding site for $PCCP^-$ within the channel: a) off currents upon removal of $PCCP^-$, b) site of action in the inner leaflet of the membrane (coinciding with the location of α_1V256/α_1T260/α_1T261, c) increase in the affinity for $PCCP^-$ after introduction of positive charges in the form of $MTSEA^+$ into M2 and d) prevention by $PCCP^-$ of the reaction of $MTSEA^+$ in different positions.

$PCCP^-$ is a rigid symmetric molecule with a diameter of approximately 10Å that can engage in interactions with metals and form hydrogen bonds with its five peripheral nitrogen atoms. These bonds can extend along the C,N-axis or at a slightly bent angle (**Figure 12B**).

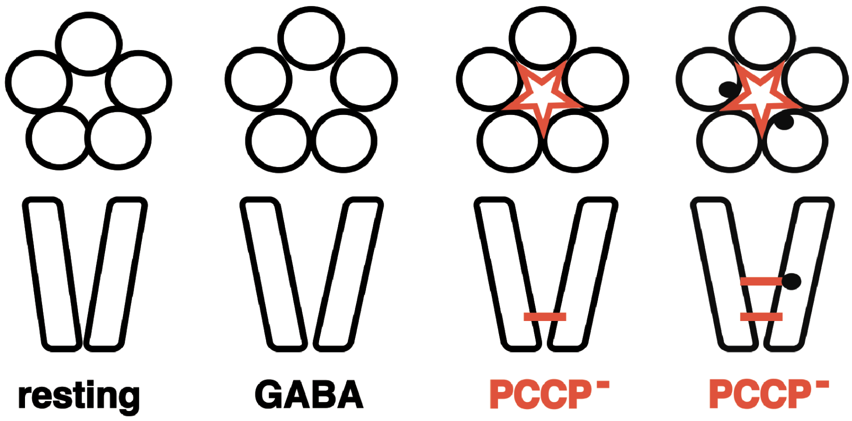

Figure 22. Hypothetical model for the mechanism of action of $PCCP^-$. Mutation of residues α_1V256 and α_1T261 to cysteine alters strongly the apparent affinity for channel inhibition by $PCCP^-$. The fact that $MTSET^+$ can only react with cysteines introduced in M2 in the presence of GABA indicates that GABA widens the pore. For the mutations α_1V256C and α_1T260C the affinity for $PCCP^-$ is strongly increased after $MTSET^+$ treatment. $MTSET^+$ reaction is prevented by $PCCP^-$. This together implies these residues in $PCCP^-$ binding. Introduction of two positive charges by reaction with $MTSET^+$ further up in the channel leads to additional binding sites for $PCCP^-$.

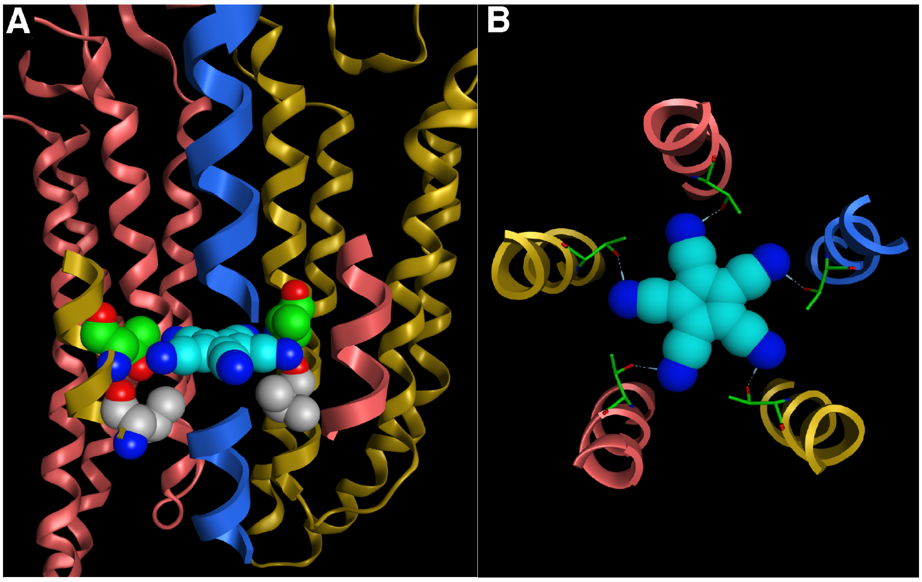

Figure 23. Molecular model of the interaction of PCCP$^-$ with GABA$_A$ receptors. A, The side view of the PCCP$^-$ docking pose from the perspective of the γ_2 subunit. The ligand and the mutated residues of the α_1 subunit, which have an impact on the affinity of the ligand, are shown in space filling representation. The 2' valines of the α_1 subunit are rendered grey; the 6' threonines of the α_1 subunit in green. The GABA$_A$ receptor is displayed in ribbon representation with α_1 subunits shown in yellow, β_2 subunits in red, γ_2 subunit in blue. The complete transmembrane domain (TMD) is shown only of the α_1 and the β_2 subunits in the back. Of the subunits in front, only a segment of the transmembrane domain 2 (TMD2) is depicted. The TMD2 of the γ_2 subunit is only partly displayed to provide a "window" through which the ligand is seen. **B**, Top view of the pose showing the symmetric molecular interactions between ligand and receptor. PCCP$^-$ (space filling) forms H-bonds (blue dashed lines) to the –OH groups of the 6' threonines (stick representation) of each of the five subunits.

We think that the most likely interpretation of our findings is that PCCP$^-$ blocks the receptor by plugging the pore at the level of α_1V256C, α_1T260 and α_1T261, adopting a position parallel to the lipid bilayer. **Figure 23A,B** depicts a molecular model of the binding site of PCCP$^-$. The ligand is in a planar position between the highly conserved 6' level threonines (α_1T260) forming H-bonds with the hydroxy groups, and the variable 2' level of α_1V256 and the homologous β_2A251 and γ_2T267. These protein-ligand contacts are consistent with the observed affinity differences for different pentamers, as the shape complementarity and surface properties of the 2' level will be unique for each homo- or hetero-pentameric receptor. The relatively slow rate of block could be due to the strong tendency of $PCCP^-$ to form H-bridges. It may be hypothesized that on the way down the channel lumen it interacts several times with the receptor.

It is interesting to compare the binding site for $PCCP^-$ to that of picrotoxin, a well-known open channel blocker. Considerably controversy exists if picrotoxin occludes the channel directly or whether it allosterically affects the channel (reviewed in [34] and references therein). Possibly the best evidence for channel occupancy comes from crystallization experiments. The crystal structure of the homo-pentameric Caenorhabditis elegans glutamate-gated chloride channel α (GluCl) shows that picrotoxin (9 Å diameter) directly occludes the pore near its cytosolic base at the 2' Thr and -2' Pro side chains [35]. In this position, the channel diameter in static condition of the crystal is 4.6 Å. These residues are homologous to P252 and V256 in the M2 of the α_1 $GABA_A$ receptor subunit. Thus, it appears that $PCCP^-$ and picrotoxin occupy sites in the lumen of the channel toward the intracellular side of the transmembrane domain that are shifted by one turn of the α-helix.

Pentasymmetric protein assemblies are not confined to Cys-loop receptors but frequently occur elsewhere in Nature, for instance in mechanosensitive ion channels, such as MscL, or bacterial toxins, such as Shiga toxin B. Indeed, symmetry-adapted inhibitors for the latter have been developed that show extremely high avidity (picomolar) due to their polyvalency [36]. More recently, polycationic blockers of voltage gated potassium channels based on a four-fold symmetric calixarenes [36] and a blocker of the heptameric Anthrax PA channel based on seven-fold symmetric β-cyclodextrin [37] have been introduced. This underscores that symmetry considerations hold considerable promise for the development of new pharmacophores. Given the prevalence of symmetric protein assemblies in Nature, it seems likely that many symmetry adapted agonists, antagonists and blockers will emerge in future years.

In conclusion, we have identified a new potent blocker of $GABA_A$ receptors through rational design rather than a massive screening effort. Our work demonstrates that symmetry considerations can contribute to the pharmacology of Cys-loop receptors. The application of other five-fold-symmetric molecular platforms including some shown in **Figure 12** to the development of high-affinity ligands for $GABA_A$ receptors as well as other five-fold symmetric ion channels such as mechanosensitive channels is under active investigation and will be reported in due course.

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2.2 Manuscript 2: Azo-propofols: photochromic potentiators of $GABA_A$ receptors.

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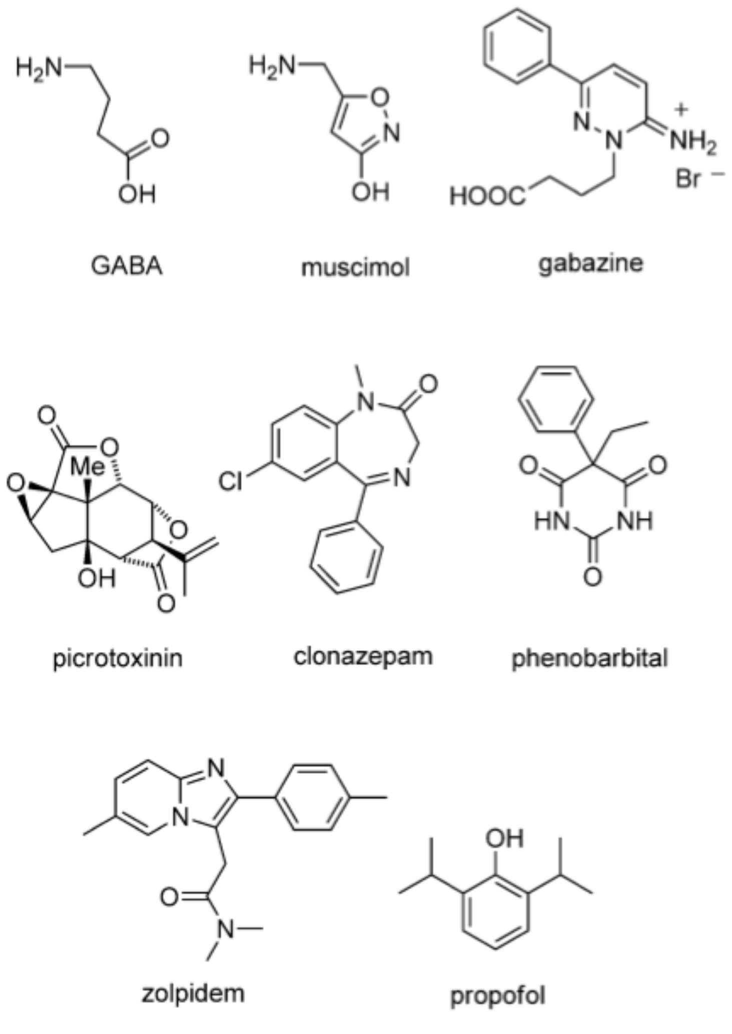
Abstract

Shine and rise! $GABA_A$ receptors are ligand-gated chloride ion channels that respond to γ-aminobutyric acid (GABA), which is the major inhibitory neurotransmitter of the mammalian central nervous system. Azobenzene derivatives of propofol, such as compound 1, increase GABA-induced currents in the dark form and lose this property upon light exposure and thus function as photochromic potentiators. Compound 1 can be employed as a light-dependent general anesthetic in translucent tadpoles.

Keywords: azo compounds, GABA receptors, ion channels, photochromism, photopharmacology.

Introduction

$GABA_A$ receptors are pentameric ligand-gated ion channels that are activated by the major inhibitory neurotransmitter in the mammalian brain, γ-aminobutyric acid (GABA).[1] Binding of GABA results in the opening of a chloride ion selective pore, thus hyperpolarizing the postsynaptic neuron and decreasing the likelihood of actionpotetial firing. As such, $GABA_A$ receptors are a prominent target for anesthetic, hypnotic, and anticonvulsant drugs (**Scheme 1**).[2, 3] While agonists, antagonists, and blockers of $GABA_A$ receptors, such as muscimol, gabazine, or picrotoxinin, respectively, have proven to be valuable research tools, their impact on human medicine has been limited. Drugs that target these receptors are dominated by allosteric modulators that potentiate, that is, increase, chloride currents elicited by the neurotransmitter. Well-established potentiators include benzodiazepines (e.g. clonazepam), barbiturates (e.g. phenobarbital), the imidazopyridine zolpidem, and the simple phenol propofol.[2] These drugs bind to distinct allosteric sites on $GABA_A$ receptors, thereby increasing the mean open time or the opening frequency of the channel. However, the analysis of their exact binding sites at a molecular level has been complicated by a lack of detailed structural data.

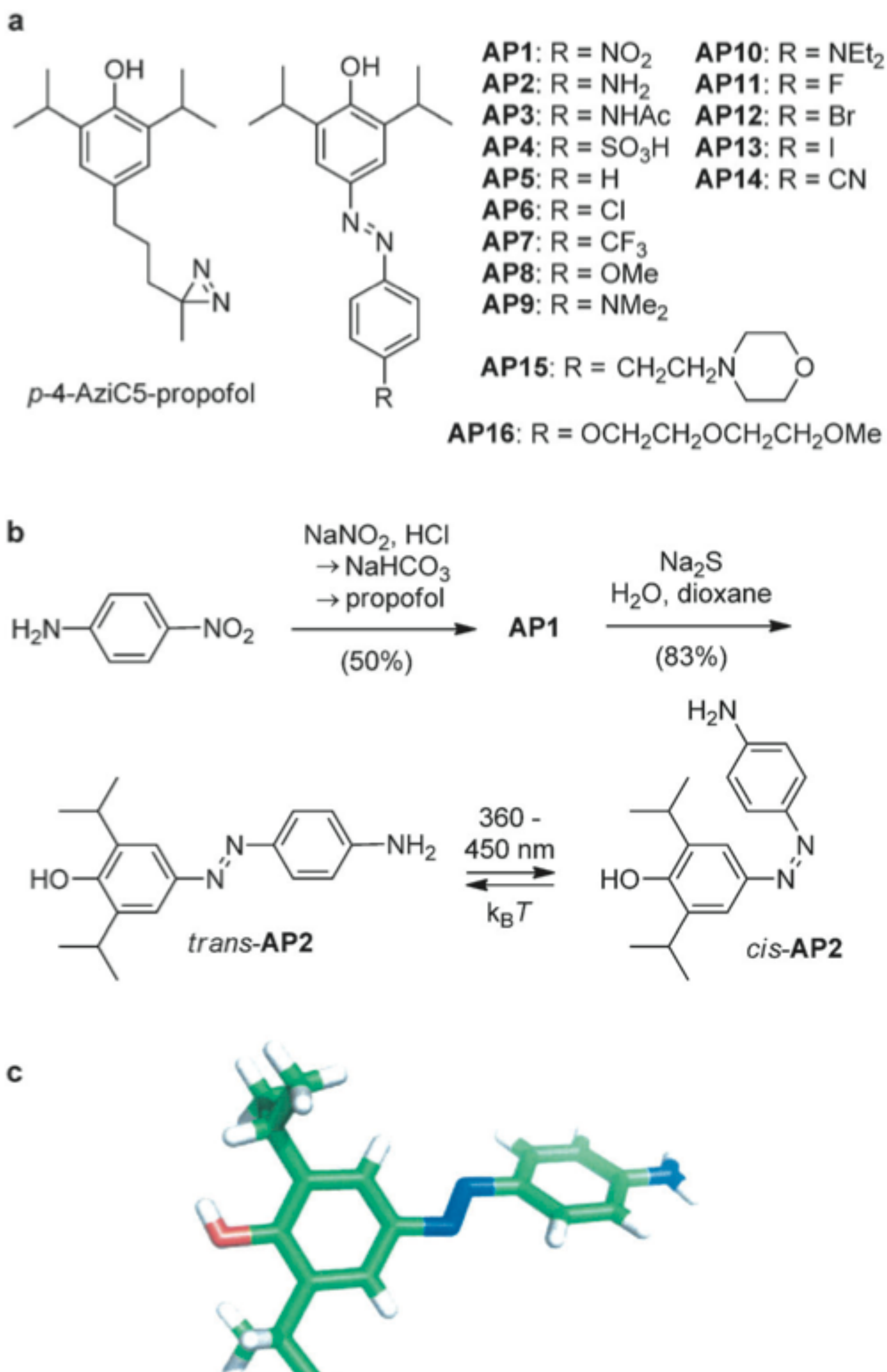


Scheme 1. Agonists (GABA, muscimol), antagonists (gabazine), blockers (picro-toxinin), or potentiators (clonazepam, phenobarbital, zolpidem, propofol) of $GABA_A$ receptors.

After its discovery in 1980, propofol has become the most widely used intravenous general anesthetic.[4] Although its mode of action has not been fully elucidated, it is commonly accepted that the anesthesia induced by this unusually lipophilic drug mostly results from potentiation of GABA-induced currents, as well as a direct activation of the chloride ion channel at high concentrations. Propofol has a rapid onset and offset of action and shows only minimal accumulation upon prolonged use. The intravenous administration of propofol is also associated with reduced postoperative nausea and vomiting.[5]

While $GABA_A$ receptors respond to a variety of ligands, they are normally not sensitive toward light. It would be fascinating to confer light sensitivity to these ion-channels, since light is unsurpassed in terms of the temporal and spatial precision it provides. This light sensitivity could be indirectly achieved by using ligands that act on the receptors but can be optically switched between an active and an inactive form. Photochromic ligands of $GABA_A$ receptors could be agonists, antagonists, or allosteric modulators. In principle, these ligands could be covalently attached as photoswitched tethered ligands (PTLs) or act as soluble photochromic ligands (PCLs).[6] Indeed, both approaches have been used to convert neuronal[7] and neuromuscular[8] nicotinic acetylcholine receptors, another type of pentameric ligand-gated ion channels, as well as ionotropic glutamate receptors[9] into artificial photoreceptors. Tethered and soluble photochromic blockers of K^+, Na^+, and Ca^{2+} ion channels have been described as well and have been used to control heartbeat,[10] pain sensation[11] and visual responses[12] in different animals with light. We now report photochromic potentiators of GABA currents that change the strength of GABA-induced currents in a light-dependent fashion.

Our program was prompted by a recent report on a photoaffinity probe based on propofol, p- 4-aziC5-propofol that underscored that a relatively large substituent in the para-position of the phenol would be tolerated and that the propofol pharmacophor would be compatible with photochemistry (**Scheme 2a**).[13]



Scheme 2. a) p-4-aziC5-propofol, a photoreactive derivative of propofol, and AP1-16, photoswitchable derivatives of propofol. **b**) Synthesis of AP1 and AP2, which is shown in its trans and cis configuration. **c**) X-ray structure of trans-AP2; C green, O red, H white, N blue.

Accordingly, we designed a series of azobenzene derivatives of propofol; in these derivatives an aryldiazene unit is directly coupled to the pharmacophor. These molecules, termed azo-propofols 1–16 (AP1–16) are shown in **Scheme 2a**.

In addition to this, the substitution of the azobezene core with electron-donating substituents greatly decreases the thermal stability of the cis isomer. Therefore, AP2 quickly reverts to its trans form once the light is switched off. Since the absorption spectra of the cis and trans isomers are very similar (see the Supporting Information), this process cannot be accelerated by irradiation with a different wavelength. Other APs studied have less favorable photophysical properties, show decreased potency (e.g. AP3, AP9, AP10), no activity at all (e.g. AP4),[14c] or unfavorable solubility and distribution (e.g. AP6).

Results

The effect of AP2 on Cl- currents was investigated with electrophysiology using $\alpha1\beta2\gamma2$ $GABA_A$ receptors expressed in Xenopus oocytes (**Figure 24**).[16] This receptor subtype represents the most prevalent form in the human brain.[17] First, the heterologously expressed $GABA_A$ receptors were exposed to GABA at a concentration eliciting 0.3% of the maximal current amplitude in combination with increasing concentrations of propofol or AP2 in the dark to compare the relative effect of the compounds. From the resulting dose– response curves, we extracted an EC_{50} value of (17.1 ± 2.9) µM for propofol and of (6.1 ± 0.4) µM for AP2 (mean ± standard error of the mean (SEM), n = 4 ; **Figure 1a**). Thus, AP2 in its dark-adapted trans form has a significantly higher affinity than propofol itself, albeit its efficacy is reduced by about two fold when compared with its parent compound.

Having established that AP2, in its dark-adapted form, has an effect on $GABA_A$ receptors, we investigated the light dependency of the current potentiation. UV/Vis light from an Ultrafire 1Watt UV LED pocket lamp (YonC Trading, Zürich; emission wavelength 390–450 nm) had no effect on the GABA response or the combined GABA/propofol response (data not shown).

Figure 24b illustrates the effect of UV/Vis light on currents elicited by the combined application of GABA and AP2. Stimulation of GABA currents by AP2 (1.5 μM) was (159 ± 25)% (mean ± SEM, n = 6). Exposure to light decreased the residual stimulation to (18 ± 3)% (mean ± SEM, n=6). Similar observations were made using a UV high power LED pocket lamp, 5 Watt (Uveco GmbH, Bruckmühl, Germany), emission wavelength 355-380 nm equipped with a CHROMA bandpass filter D365/ 10x, to limit light emission to 360-370 nm.

The possibility to use these different light sources reflects the broad absorption spectrum of AP2. Owing to redistribution of the hydrophobic compound AP2 into egg yolk, the rate of photoswitching could not be determined in Xenopus oocytes. For this purpose, we expressed $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors in HEK cells and performed experiments using the whole-cell patch- clamp technique. GABA was co-applied with AP2.

Subsequently, the perfusion was stopped to prevent arrival of new trans-AP2 during the measurement, and the cells were exposed to the light. The current amplitude decreased rapidly and increased again upon turning off the light source. Current traces were fitted with a mono-exponential function. The time constant t amounted to (1.1 ± 0.4) s (mean ± SD, n = 7) for the trans-to-cis transition and (2.0 ± 0.7) s (mean ± SD, n = 6) for the cis to trans transition.

Next, we investigated anesthetic activity and photoreversibility of both propofol and AP2 in a small animal model, albino Xenopus laevis tadpoles. Groups of animals were placed in aqueous solutions containing either propofol or AP2 and tested every five minutes for loss of righting reflexes (LORR), a standard assay for anesthesia.[13]

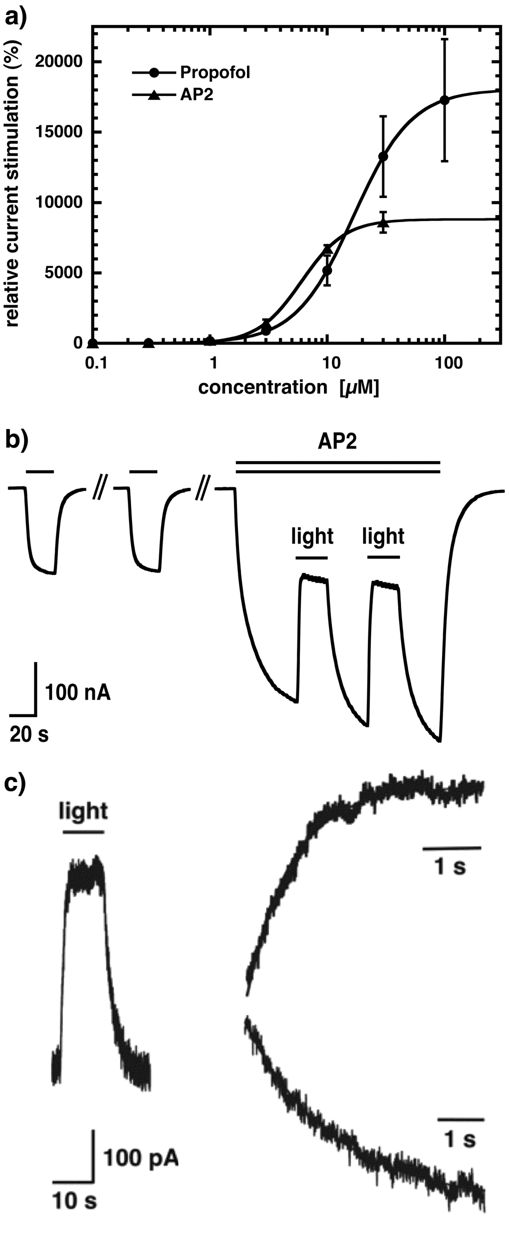


Figure 24. a) $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were expressed in Xenopus oocytes. Currents were activated with a concentration of GABA eliciting 0.3% of the maximal current amplitude (EC 0.3) with increasing amounts of either propofol or AP2. Mean ± SEM of four experiments is shown. **b)** GABA (1µM) was applied repetitively until a stable current response was observed. Co-application of AP2 (1.5 µM) with GABA resulted in current potentiation. During co-application, the oocytes was exposed to a light source emanating 390-450 nm light. As a consequence, current stimulation rapidly decreased until it reached a steady level. When the light-source was turned off, the amplitude increased again. This procedure was repeated. This experiment was repeated independently six times using different oocytes. **c)** $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were expressed in HEK cells. GABA (0.5 µM) was co-applied with AP2 (5 µM). Subsequently, the perfusion was stopped and the cells were exposed to the light source. The inward current amplitude decreased rapidly and increased again upon turning off the light source (trace left). Current decrease (trace top right)

Steady-state LORR results were observed at 10 min for propofol and 25 min for AP2. After 30 min in drug solution, each animal was exposed for five to ten seconds to 360–370 nm bandpass filtered UV light (details in the Supporting Information) while retesting for LORR. Propofol alone produced LORR with an EC_{50} of 1.3 μM (**Figure 25a**).

Illumination induced vigorous swimming activity in unanesthetized tadpoles (thus suggesting that illumination represents a noxious stimulus) and a small rightward shift in the corresponding plot of LORR versus propofol concentration was observed (EC_{50} ca. 2.0 μM ; **Figure 25a**). AP2 alone produced LORR with an EC_{50} value similar to that of propofol (1.1 μM ; **Figure 25b**). However, illumination produced a large rightward shift in the AP2 EC_{50} value to 4.6 μM (**Figure 25b**).

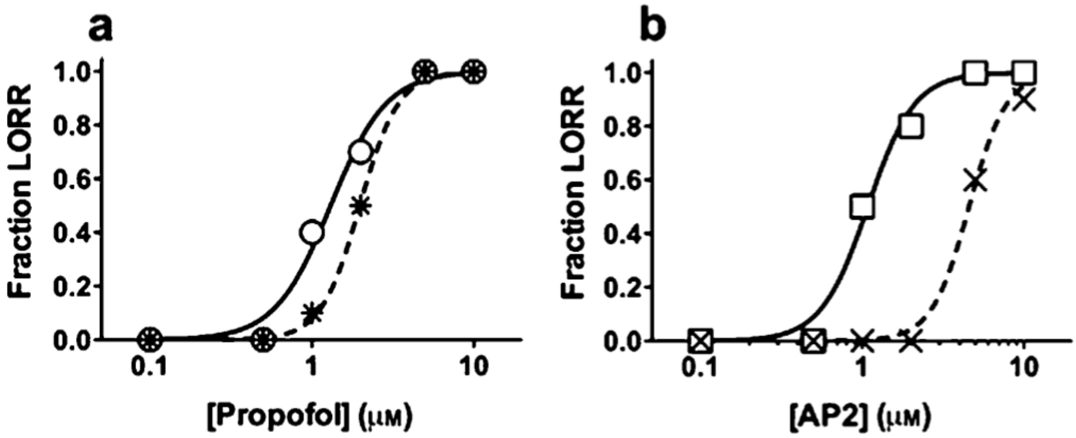


Figure 25. **Light-dependent anesthesia in tadpoles.** Loss of righting reflexes is plotted against aqueous anesthetic concentration, overlayed with logistic fits. Each point represents data from ten animals. **a)** 360– 370 nm light, an apparently noxious stimulus in Xenopus tadpoles, produces a small rightward shift in propofol-dependent loss of righting reflexes (LORR) from (1.1 ± 0.1) μM (circles) to (2.0 ± 0.1) μM (stars). **b)** 360–370 nm light shifts the AP2-dependent LORR curve to the right from (1.1 ± 0.1) μM (squares) to 4.6 ± 0.2 μM (crosses). This larger shift is due to photoisomerization of AP2.

All animals recovered from anesthesia when returned to water alone. In an independent set of experiments (see video in the Supporting Information), propofol (3 μM) produced LORR in all tadpoles with or without light, whereas in AP2 (3 μM), all animals showed LORR without light and all spontanously righted themselves and swam during illumination with UV light.

The photoreversibility of both AP2-induced $GABA_A$ receptor modulation and its anesthetic action in animals supports the hypothesis that anesthesia caused by AP2 and propofol is largely mediated by $GABA_A$ receptors. However, evidence also implicates other targets, including HCN1 channels (hyperpolarization-activated cation channels),[18] in propofol's anesthetic actions. The examination of the effects of AP2 on these other targets and the investigation of the photoreversibility of the modulation of these targets might help to further elucidate their roles in the pharmacology of general anesthesia.

Conclusion

In summary, we have developed photoswitchable versions of propofol that allow the indirect optical control of $GABA_A$ receptors. Functionally, our compounds differ from previously introduced PCLs, because they act as photochromic potentiators rather than photochromic agonists, antagonists, or channel blockers. Application of our lead compound, AP2, in the dark potentiates GABA-induced Cl^- currents, which can be reversed upon irradiation with violet light. The ability of azo-propofols to control neural systems has been demonstrated, since AP2 functions as a light-dependent anesthetic in translucent tadpoles. Future work will address the usefulness of azo-propofols in other systems, such as brain slices and retinas lacking innate photoreceptors, wherein photochromic potentiators could restore visual responses through their action on neurons expressing $GABA_A$ receptors.

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[15] AP2: C18H23N3O, Mr = 297.395 g mol-1, red block, 0.14 x 0.25 x 0.30 mm, monoclinic, P21, a = 10.5454(3), b = 9.5435(3), c = 17.0651(4) Å, a = 90, β = 90.0955(16), γ = 90°, V = 1704.28(8) Å3, Z = 4, ρ = 1.159 g cm-3, μ (MoKα) = 0.073 mm-1, MoKα radiation (λ = 0.71073 Å), T = 200 K, 2θmax 55.02°, 13750 refls. , 4143 independent, 3271 with I ≥ 2σ(I), Rint = 0.042, mean σ(I)/I = 0.0392, 429 parameters, R(Fobs) = 0.0432, Rw(F2) = 0.1097, S = 1.014, min. and max. residual electron density : -0.21, 0.15e Å-3; data collection by means of a Nonius KappaCCD diffractometer equipped with a rotating anode generator (w-scans), structure solution by direct methods with SIR97, structure refinement with

SHELXL-97, O- and N-bonded H atoms have been refined freely, C-bound H atoms have been added geometrically treated as riding on their parent atoms. CCDC 890176 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc. cam.ac.uk/data_request/cif.

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Author contribution

S. J. M. performed functional experiments of the azo-propofol derivatives (APs) in the oocyte, M. S. performed the synthesis of the APs, V. C. performed kinetic experiments in HEK-cells doing whole-cell patch-clamp recordings. E. P., D. E. R. performed in vivo 93 experiments in tadpoles, M. S., S. J. M., S. A. F., E. S., and D. T. wrote the manuscript.

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3 Discussion and Outlook

3.1 A novel insecticide?

We identified an aromatic monovalent anion with five-fold symmetry ($PCCP^-$) as an inhibitor of rat $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. Our work should be compared to other reports on non-competitive inhibitors of the $GABA_A$ receptor. Studies carried out with negatively charged sulphated steroids, such as pregnenolone sulphate showed an uncompetitive inhibition of the $GABA_A$ receptor-mediated current (Shen et al., 2000; Eisenman et al., 2003) through steady-state allosteric effects (Shen et al., 2000; Akk et al., 2001; Eisenman et al., 2003). Interestingly, pregnenolone sulphate (PS) antagonism was dramatically reduced by mutation in the M2 residue V256 of the α_1 subunit while the homologous mutations of the β_2 subunit (A252S) or the γ2L subunit (S266A) had no effect. Therefore the authors implied that α_1V256 is involved in a conformational change underlying block by pregnenolone sulphate, instead of forming part of the binding site for PS (Akk et al., 2001). Another study reported that hydrophobic anions, as dipicrylamine (DPA), unrelated to neurosteroids strongly antagonize $GABA_A$ receptor function in the same manner as PS (Chisari et al., 2011), including sensitivity to the V256 point mutation in the M2 of the α_1 subunit. As this uncompetitive and membrane-potential independent inhibition is shared by a wide variety of hydrophobic anions and amphipatic compounds (Chisari et al., 2010) and at sufficiently high concentrations, even uncharged neurosteroids exhibit this mechanism (Wang et al., 2002) the authors suggested that the potent antagonism of channel function by these different compounds was due to a possible plasma membrane modification that also leads to a change in membrane capacitance (Chisari et al., 2011; Mennerick et al., 2008). While these agents are hypothesized to act in an allosteric fashion we have strong evidence for a direct action of $PCCP^-$ on the transmembrane segment M2 of $GABA_A$ receptors.

PCCP$^-$ induces open-channel block, characterized by an apparent desensitization of the current and an off-current. As expected this block becomes more prominent with increasing agonist concentrations. Interestingly, the block is voltage-dependent indicating an action in the inner leaflet of the membrane. Action of PCCP$^-$ is not affected by the substitution of the β_2 subunit with β_1 or by the presence of δ subunit. We identified, through a mutation scan, several amino acid residues involved in the formation of the binding between PCCP$^-$ and $GABA_A$ receptor. The cysteine mutants α_1V256C$\beta_1\gamma_2$ and α_1T261C$\beta_1\gamma_2$ both showed 30-20 fold decrease in the apparent affinity of PCCP$^-$ to inhibit currents elicited by GABA. To validate our finding we also used the substituted-cysteine-accessibility method where a positively charged reagent MTSET$^+$ reacts covalently with the cysteine residues of the mutated subunits (Akabas et al., 1992, 1994). Covalent reaction of MTSET$^+$ was prevented in the presence of PCCP$^-$. The effect of PCCP$^-$ was potentiated after treatment with the cysteine reactive reagent MTSET$^+$. The introduction of two positive charges (one for each of the α subunits) by MTSET$^+$ compound, may favour the interaction of the channel with the negatively charged PCCP$^-$ and this is possible only if PCCP$^-$ enters the channel. Similar studies on the open channel blocker picrotoxin indicated a possible interaction between picrotoxin and α_1V256 and α_1T261 both facing the lumen of the channel, although the authors could not exclude a possible allosteric interaction through another binding site outside of the lumen (Xu et al., 1995). The crystal structure of the homo-pentameric Caenorhabditis elegans glutamate-gated chloride channel showed that picrotoxin directly occludes the chloride channel at positions corresponding to P252 and V256 in the M2 of the the rat α_1 receptor subunit (Hibbs et al., 2011). Our data indicate that PCCP$^-$, acts one turn of the α–helix shifted into M2 as compared with to picrotoxin. All crystallized cys-loop receptors have a predicted lumen diameter significantly smaller than open channel blockers. How can PCCP$^-$ with a diameter of approximately 10Å sit

into the channel? As indicated above the GluCL channel crystallized in the presence of picrotoxin shows picrotoxin at the 2’ position (0' position at the predicted membrane surface), where the diameter measures 4.6 Å (Hibbs et al., 2011). This may be explained by the fact that the surface of the channel is not smooth and very flexible. We think that $PCCP^-$ binds between the 2’ and 6’ position by plugging the pore in a parallel position to the lipid bilayer due to the H bonds formed with the hydroxy groups of the highly conserved 6’ α_1T260C and at 2’ α_1V256C. The predicted diameter in all crystallized cys-loop receptors is in that position is 6-9 Å. A location of the binding site in the inner leaflet is also predicted by the membrane potential dependence of the block by $PCCP^-$. In insect $GABA_A$ receptors RDL a point mutation of the Ala302 to Ser (homologous to rat α_1V256) conferred resistance to picrotoxin (Hosie et al., 1995). Interestingly, $PCCP^-$ inhibits these mutated channels indicating a potential use as insecticide. Chemical modification of the $PCCP^-$ structure will hopefully result in higher affinity inhibitors
A collaboration with a company resulted in promising observations in insects. The collaboration came to a halt as we were forced to publish our data.

3.2 Photopharmacology

In a second study we characterised a light-switchable modulator of $GABA_A$ receptors. While $GABA_A$ receptors respond to a variety of ligands, they are normally not sensitive toward light. This light sensitivity could be indirectly achieved by using ligands that act on the receptors but can be optically switched between an active and an inactive form. We tested an azobenzene derivative of propofol where an aryldiazene unit is directly coupled to the pharmacophor. This molecule is termed azopropofol (AP2). First, we compared the relative effect of propofol and AP2 on the $GABA_A$ receptor in the dark to compare the relative effects. AP2 in its dark-adapted trans form has a significantly higher affinity than propofol itself, which its efficacy is reduced by about two fold. Due to redistribution of the hydrophobic compound AP2 into egg yolk, the rate of photo-switching could not be determined in Xenopus oocytes. For this purpose, we studied the switching kinetics in patch clamp experiments using the whole-cell patch clamp technique. The current amplitude decreased rapidly and increased again upon turning off the light-source allowing an optical control of the $GABA_A$ receptors.

In the past optogenetic manipulations have been used to dissect neuronal functions. These manipulations included expression in neuronal tissue of photosensitive ion channels. Photopharmacology avoids genetic manipulation and adaptive phenomena observed after expression of a new gene. AP2 may be employed in brain tissue slices where one could selectively irradiate neurons or even single synapses with high temporal resolution. Furthermore, there might be a potential application in human brain surgery. It might be possible relieve narcosis locally by irradiation of part of the motor cortex and test e.g. motility properties of the patient.

4 Additional project: GABA in liver

4.1 Introduction

As described in chapter 1, $GABA_A$ receptors are also found in a wide range of peripheral tissues, including liver and several cancer tissues. However their precise function in non neuronal cells is in many cases still unknown.

The liver is the body's largest single organ. It has four major functions: metabolism and synthesis, excretion, storage, and the detoxification of potential poisons. Sympathetic, parasympathetic and peptidergic fibers are responsible for the liver innervation. In the human liver, nerve endings are located in the hepatic lobules (Ueno et al., 2004), which consist of hepatocytes and non-parenchymal cells. Hepatocytes occupy 80% of the liver mass, parenchima cells occupy only about 6.5%. Hepatocytes are arranged as cellular cords with a radial disposition that converges towards the centrilobular vein, being separated by sinusoidal capillaries. Between hepatocyte cell cords and sinusoid capillaries there is an interstitial space, a perisinusoidal called a Disse space. This space is formed by a fine network of reticulin fibers, a support for the sinusoids, non myelinated nerve fibers and mesenchymal type cells (Crişan and Mureşan, 2004). Most hepatocytes are not directly innervated but there is an indirect mechanism for transmitting nervous inflow. One such mechanism is the intercellular communication carried out between adjacent hepatocytes via specific channels known as gap type junctions (GJ), which allow the passage of ions and small molecules (Shimazu, 1996). Hepatocytes are multifunctional epithelial cells, with the main functions to regulate trans-cellular solute transport, processing of metabolites and synthesize and export of numerous important proteins. During liver regeneration, hepatocytes initiate cell proliferation, maintain metabolic function of the liver, secrete interleukin-6 (IL-6), proteases, protease inhibitors and hepatocyte growth factor (Zheng et al., 2009). Many functions are carried out by anion channels. For example a chloride channel, which belong to the

CLIC channel family is important in cell volume regulation, in the control of membrane potential and trans-cellular transport of hepatocytes (Graf and Haussinger, 1996). The presence chloride channels correlates to the membrane potential of -30 to -40 mV (Moule and McGivan, 1990).

The $GABA_A$ receptor seems to be expressed in hepatocytes. Specifically, expression of β_3 and ε $GABA_A$ receptor subunits has been described. β_3 subunit down-regulation in hepatocytes has also been associated with a decrease of the hepatocyte membrane potential and an increase of the cell proliferation. In particular, the β_3 subunit is down-regulated in liver carcinoma. Carcinoma cell lines transfected with β_3 have been reported to be less aggressive in vivo than non transfected cells (Sun et al., 2002; Minuk et al., 2007).

We planned to investigate the expression and function of the hepatocyte $GABA_A$ receptor in a model system (immortalized human hepatocytes) and healthy and cancerous primary hepatocytes. Additionally, we were interested in the localization in healthy human tissue.

4.2 Materials and Methods

Material. The immortalized human hepatocyte cell line (IHH) (Nguyen et al., 2005) was kindly provided by Dr. J.F. Dufour; primary hepatocytes and liver tissues were obtained from Dr. D. Stroka. A mouse monoclonal antibody bd17, specific for β2/β3 subunit was kindly provided by Hoffmann-La Roche; the polyclonal rabbit antibody (AP2789c) against the human β3 $GABA_A$ receptor subunit was from ABGENT (San Diego, CA 92124), a mouse monoclonal antibody (AB98968) specific for the amino acids 370-433 of human β3 $GABA_A$ receptor subunit was from ABCAM (Cambridge, UK), the mouse monoclonal against Actin protein (sc-69879) was from Santa Cruz Biotechnology (Texas, USA), a rabbit polyclonal antibody against ε protein was purchased by Aviva system Biology, the rabbit polyclonal against MRP2 protein (4446) and the rabbit monoclonal antibody against E-Cadherine (3195) were from Cell Signaling (Allschwil, Switzerland).

Cell culture. The immortalized human hepatocytes were grown in Dulbeco's minimum essential medium F12 (Life technology, Zug, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin, and 50 mg/mL Gentamycin, 10 mg/mL Insulin-transferrin-selenite and 2mg/mL Dexamethasone in a humidified, 37°C incubator in an atmosphere of 95% air-5% CO_2. Primary human hepatocytes were isolated from the resected liver tissue of consented patients undergoing liver surgery. Human hepatocytes were enzymatically dissociated from human liver samples using a two-step enzymatic microperfusion technique with collagenase and kept on ice in suspension (Strain, 1994). Hepatocytes were subsequently seeded in plastic dishes coated with rat-tail collagen (25 $\mu g/cm_2$) at a density of 130,000 viable cells/cm^2 and cultured in Dulbeco's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin, and 1 µM dexamethasone. After overnight culture, the medium was replaced by serum-free Williams' E medium supplemented with 100 nM hydrocortisone, 50 U/ml penicillin, 50 µg/ml streptomycin, and solution ITS + 1, containing insulin (5 µg/ml), transferrin (2.75 µg/ml), and selenium (25 ng/ml) (Sigma, Buchs, Switzerland). The medium also was supplemented with 250 µg/ml bovine serum albumin and 2.35 µg/ml linoleic acid. In the following days after serum deprivation, the human hepatocytes were kept in serum-free medium and harvested at different time points for protein and electrophysiological analysis.

RT-PCR and qPCR for detection of $GABA_A$ receptors subunits. Total RNA was extracted from 20%, 40%, 60%, 80% cell confluence, by the commercially available TRIZOL method (Invitrogen, Carlsbad, CA). To determine quality and integrity of the RNA the ratios of the absorption at 280 and 260 nm was determined; RNA was stored at -80° C until further analysis. Reverse transcription of 1 μg RNA was performed with random hexamer primers using the Applied Biosystems (Zug, Switzerland) reverse transcription kit according to the manufacturer's instructions.

TABLE 2. Gene-specific PCR and qPCR primers for Human $GABA_A$ receptor subunits

Species / Gene	Sequence [5'-3']	Accession number	Anneling temperature (°C)	Fragment lenght [bp]
Homo Sapiens				
α_1	TCGTCACCAGTTTCGGACC	NM_000806.5	58	902
	GGTTGCTGTTGGAGCGTAA			
α_2	TTCACAATGGGAAGAAATCAGTAG	XM_006714002.1	60	722
	TGCATAAGCGTTGTTCTGTATCA			
α_3	GGAAGTGGCACAGGATGGTTC	NM_000808.3	60	658
	GTTGTAGGTCTTGGTCTCAGTCGG			
α_4	TGAAATTCGGGAGTTATGCCTATC	NM_000809.3	60	750
	GGCTGAATGGGTTTGGACTG			
α_5	CACCATGCGCTTGACCATCTCT	NM_000810.3	60	826
	GCCGAACAAGACTGGGAATA			
α_6	TGAGGCTTACCATCAATGCTGA	NM_000811.2	60	764
	GACAGGTGTTGATTGTAAGATGGG			
β_1	GTTCTCTATGGACTCCGAATCACA	NM_000812.3	60	603
	ATTGGCACTCTGGTCTTGTTTG			
β_2	AGCTTAAGAGAAACATTGGCTACT	NM_000813.2	58	643
	CGATCTATGGCATTCACATCA			
β_3	ATGGGCTCAGAATCACCA	NM_000814.5	58	250
	GATAGGCACCTGTGGCGA			
γ_1	GTGTTTTGCAGCCTTGATGG	NM_173536.3	58	284
	TGGCAATGCGTATGTGTATCCT			
γ_2	AAGTCCTCCGATTGAACAGCAACA	NM_000816.3	60	605
	CGCTGTGACATAGGAGACCTT			
γ_3	ACACTCCTGCCCGCTGATT	XM_006724812.1	58	767
	TGTCTATGTGAATACGCCCTTTCC			
δ	TCACCATCACCAGCTACCACTTCA	NM_000815.4	58	654
	GGGCGTAAATGTCAATGGTGTC			
ε qPCR	CTCTCGCATCCTGAACACTATCC	NM_004961.3	60	104
	GGCTGTTGACGGAGATCTCAA			
β_3 qPCR	GACAAGGCTGTTACCGGAGT	NM_000814.5	60	127
	CGAAAGCTCAGTGACAGTCG			

The oligonucleotide primers for the RT-PCR and qRT-PCR were designed against human $GABA_A$ receptor sequences by using Oligo 4.0. The sequences of the human $GABA_A$ receptor subunits oligonucleotide primers are show in the **Table 2**.

The RT-PCR amplification was carried out in 35 cycles of denaturation at (92°C, 12 sec), anneling (55°C, 15 sec), and elongation (68°C, 1min) and with an additional 2 min final extension at 72°C. Finally 10 µl of the PCR products were run on 2% agarose gels. qRT-PCR was performed using Power SYBR Green Master Mix (Promega, Foster City, CA, USA) in a final volume of 25 µl and a final primer concentration of 150 nM. qRT-PCR were carried out on a ABI Prism 7500 real-time PCR detection system (Applied Biosystems). For each gene a standard curve was generated using a plasmid containing β3 cDNA or ε cDNA to determine detection limits and efficiencies for each primer pair. Amplified products underwent melting curve analysis to specify the integrity of amplification. To remove non-specific signals, a higher fluorescence acquisition temperature was chosen (**Table 2**), according to the melting curve that was recorded for each reaction. In all studies, the arithmetic mean of the C_T values (the cycle number at which logarithmic plots cross calculated threshold lines) of β−actin, was used to normalize the abundance of target mRNA to that of total mRNA. Calculations were done using the software KaleidaGraph.

SDS-gel electrophoresis and immunoblotting techniques. Cells were harvested by scraping into a protease inhibitor mixture consisting of (in mM) 10 HEPES, 3 EGTA, 1 PMSF, 10µL Protease Inhibitor Cocktail solution (Sigma, Buchs, Switzerland). Protein concentrations were measured by using the BCA protein assay (Thermo Scientific). Total protein extracts (10 µg) were separated on 10% polyacrylamide-SDS gels and electroblotted to nitrocellulose membranes. Membranes were blocked with 5% skim milk in Tris buffered saline (0.02M Tris base pH 7.6) for 1h at room temperature and incubated with the rabbit anti-human $GABA_A$-β_3/β_2 50 µg/ml (bd17) receptor antibody or the monoclonal mouse anti-human $GABA_A$-β_3 1 μg/ml (AB98968), overnight at 4°C. Bands were detected with a horseradish peroxidase labeled secondary antibody (anti mouse P0260, Dako, 1:1000 and the anti rabbit W401B, Promega, 1:20000) catalysed chemiluminescence reaction (Thermo Scientific, Switzerland). Controls included calf brain membrane protein and protein from the HEK cells (American Type of Culture Collection, MD, USA, CRL 1573) transfected with β_3 $GABA_A$ subunit cDNA using the calcium phosphate precipitation technique (Chen and Okayama 1987).

Immunohystochemistry. Human liver samples from tumour-free resection margins or non-tumor liver were obtained already embedded in Tissue –Tek™ Cryo OCT from Dr. Deborah Stroka. Tissue was cut into 6 μm sections with Leica Microtome and fixed for 5 min with 4% paraformaldehyde, then the slides could be frozen at -20°C until further use. Sections were blocked with 0.2 M glycine for 5 min and then blocked with 5% normal goat serum /0.05% Casein/PBS-Tween20 (PBST) for 1h. Primary antibody (mouse monoclonal 1:1000 and rabbit polyclonal anti-β_3 1:10, anti- E-Caderine 1:50, anti-MRP2 1:200) in blocking solution were incubated overnight at 4°C. Sections were washed 3x times in 1X Phosphate Buffered Saline Tween-20 (PBST). The secondary antibody (Alexa Fluor® 488 and 594 conjugated goat anti-rabbit polyclonal secondary antibody 1:1000) were incubated for 1h at RT in blocking solution. Sections were mounted with Eukitt (Kindler, GmbH). The signals were detected with a confocal microscope (Olympus).

Electrophysiology. The whole-cell patch-clamp technique was used to record currents activated by GABA (10 μM) from IHH cells voltage-clamped at 0 mV. GABA was applied using a perfusion system consisting of glass reservoir connected to a Warner Perfusion solenoid mini valve control system (Harvard Apparatus). The recording chamber was continuously perfused (5 ml/min) with an extracellular solution comprized of (in mM) NaCl, 146; KCl, 5; $MgCl_2$, 4; $CaCl_2$, 1; glucose, 5; and HEPES–NaOH, 10 (pH 7.4). The electrode solution contained (in mM): Cs Methyl sulfonate, 125; $MgCl_2$, 2; EGTA, 0.5; ATP (Mg^{2+} salt) 2; and HEPES–CsOH, 10 (pH 7.4). Experiments were performed at room temperature (20–24 °C). The gap junction blocker Octanol (1 mM) (Contreras et al., 2002) obtained from Sigma (St. Louis, MO) was added to the extracellular solution. This combination of external and internal solution produced a chloride equilibrium potential of -60 mV. Measurements were performed using an EPC-10 amplifier (HEKA, Lambrecht/Pfalz, Germany). The currents filtered with a 0.1 kHz Bessel filter and sampled at 0.5 kHz.

4.3 Results

mRNA expression. mRNA expression was investigated in the immortalized human hepatocyte cell line (IHH) (Nguyen et al., 2005) in three different independent total RNA isolations to see if these cells present a similar expression pattern of $GABA_A$ receptor subunits as healthy hepatocytes. The results of the end point RT-PCR in IHH cells showed the presence of α3, β3, β2, ε $GABA_A$ receptor subunits (**Figure 26**).

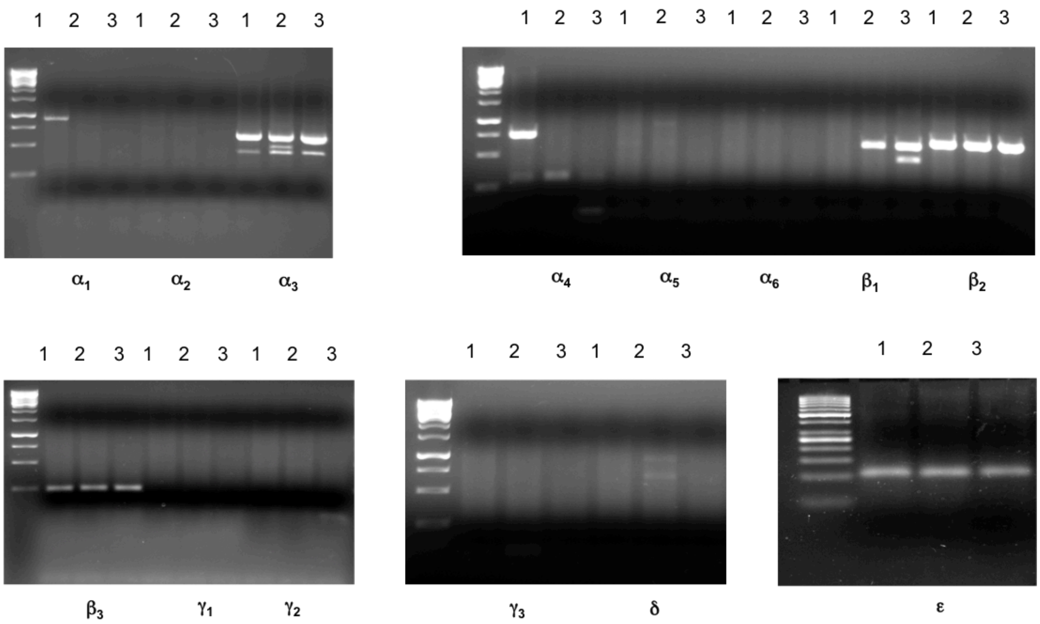


Figure 26. $GABA_A$ receptor subunit isoform mRNA expression in three RNA isolations from IHH cells at 100% confluency. The experiment was carried out three additional times with similar results.

As it has been reported that expression of β3 inversely correlates rate of with cell division, we investigated whether β3 and ε $GABA_A$ receptor subunits expression correlates with the degree of confluency. mRNA abundance was measured by real time qPCR in IHH cell at 40% confluence corresponding to dividing cells and 100% corresponding to non dividing cells. Relative mRNA levels of β3 and ε were similar at 40% confluency and at 100% confluency (**Figure 27).**

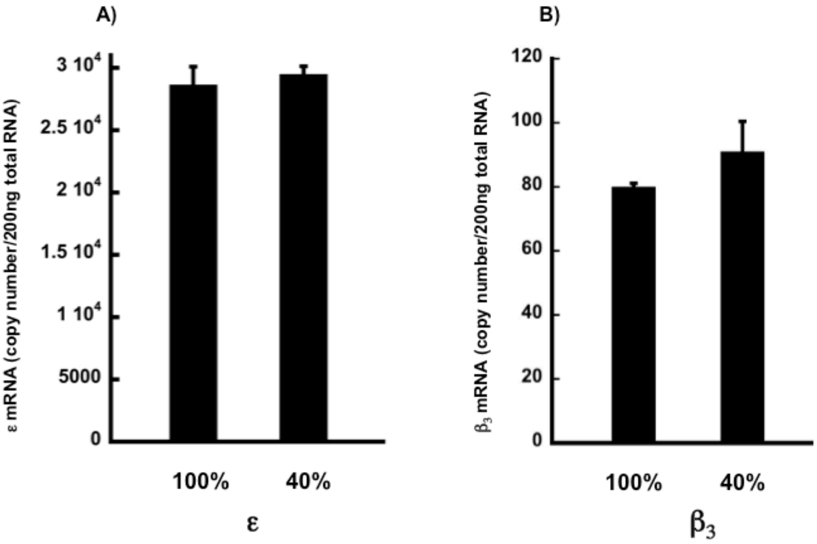


Figure 27. (A-B) qPCR analysis of ε and $\beta_3$and $GABA_A$ receptor subunits in IHH cells at 100% and 50% confluency.

4.3.1 Protein expression.

At the protein level β_3 subunits were detected in IHH cells using two different antibodies for β_3: a mouse monoclonal antibody bd17, specific for β_2/β_3 subunit and a mouse monoclonal antibody AB98968, specific for the amino acids 370-433 of human β_3 $GABA_A$ receptor subunit. The specificity of the antibodies was confirmed by Western Blot analysis of lysates of COS-7 cells transfected with recombinant β_3 subunit. All antibodies described above recognized a band of 55KDa that was specific for the transfected cells (**Figure 28**).

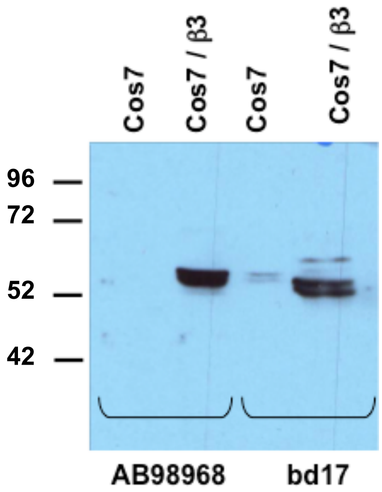


Figure 28. Western blot analysis in COS7 cells transfected with β_3 plasmid to check the antibody specificity for β_3 GABA$_A$ receptor subunit protein expression.

Protein extracts from both liver tissue and IHH cells showed a band of 55KDa using both bd17 and AB98968 antibodies (**Figure 29**).

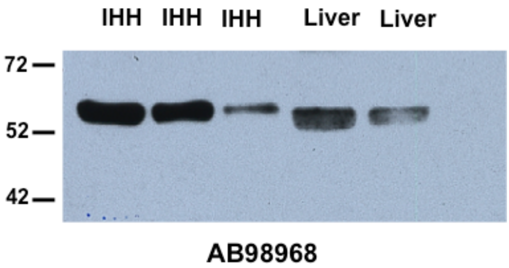
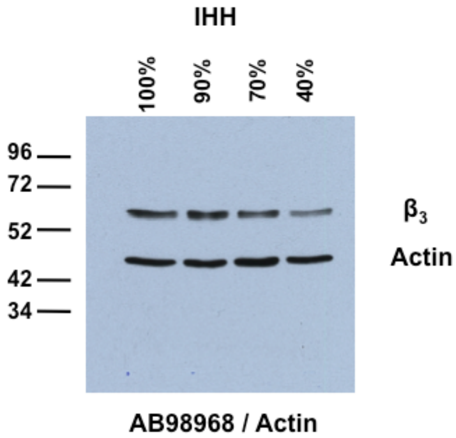


Figure 29. Protein expression of β_3 in IHH cells and liver tissue.

To investigate whether β_3 protein expression in IHH cells correlates with proliferation, cell lysates were harvested at 4 different degrees of confluency (40%, 70%, 90% and 100%) and analysed by Western Blot.

The protein abundance was quantified by densitometric image analysis of the blots. In 40% confluent cells, β_3 protein levels amounted to approximately 20% as compared to the over-confluent cells, (**Figure 30**).

In order to demonstrate ε subunit expression, we used a rabbit polyclonal antibody against ε protein. We observed a band of 57KDa in IHH cell, primary hepatocytes and liver tissue (**Figure 31**).



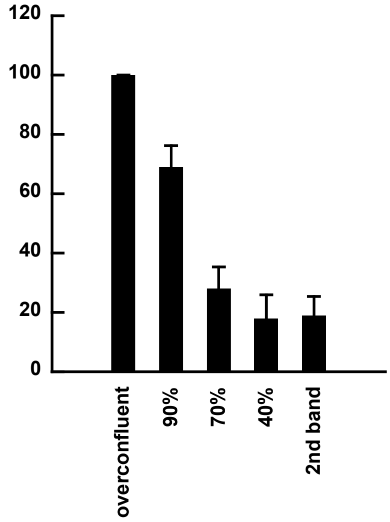


Figure 30. Expression levels of the β_3 subunit were determined by western blot analysis in IHH cells grown at different degree of confluency: 40%, 70%, 90% and overconfluent. In all the samples the same amount of protein was loaded. Detection of Actin served as a loading control (A). The protein abundance was quantified by image analysis of the blots. Band intensity was normalized tot he overconfluent values. The β_3 subunit is downregulated in 40% confluent cells as compared with overconfluent cells. Data are given as mean ± SEM (6 independent experiments).

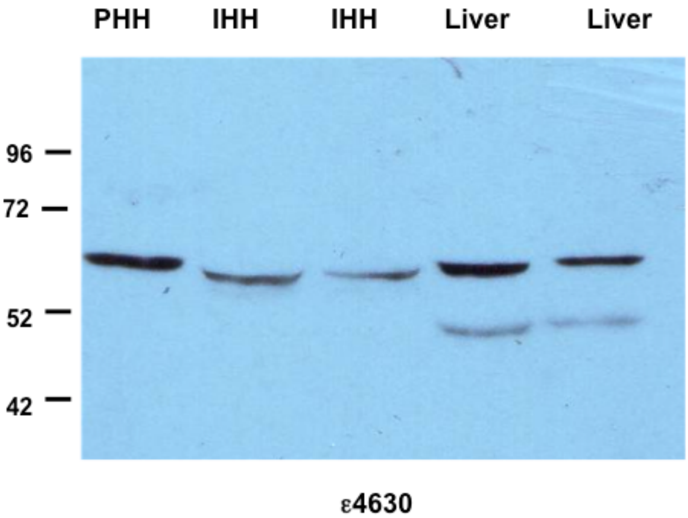


Figure 31. Protein expression of ε subunit in IHH cells and liver tissue

4.3.2 Localization (Immunohistochemistry)

We localized the β_3 subunit in healthy freshly frozen liver tissues using immunofluorescence on a confocal microscope. We used two antibodies specific for β_3: monoclonal mouse antibody (AB98968) and polyclonal rabbit antibody (AP2789c). Both antibodies showed a basolateral membrane staining for β_3 subunit in healthy liver tissue. Double labelling with apical markers, an antibody specific for the multidrug resistance protein (MRP) 2 showed a precise complementary apical staining with β_3 basolateral staining. (**Figure 32**). The commercial antibody specific for ε subunit used in western blot failed to stain the antigen in immunohistochemical sections.

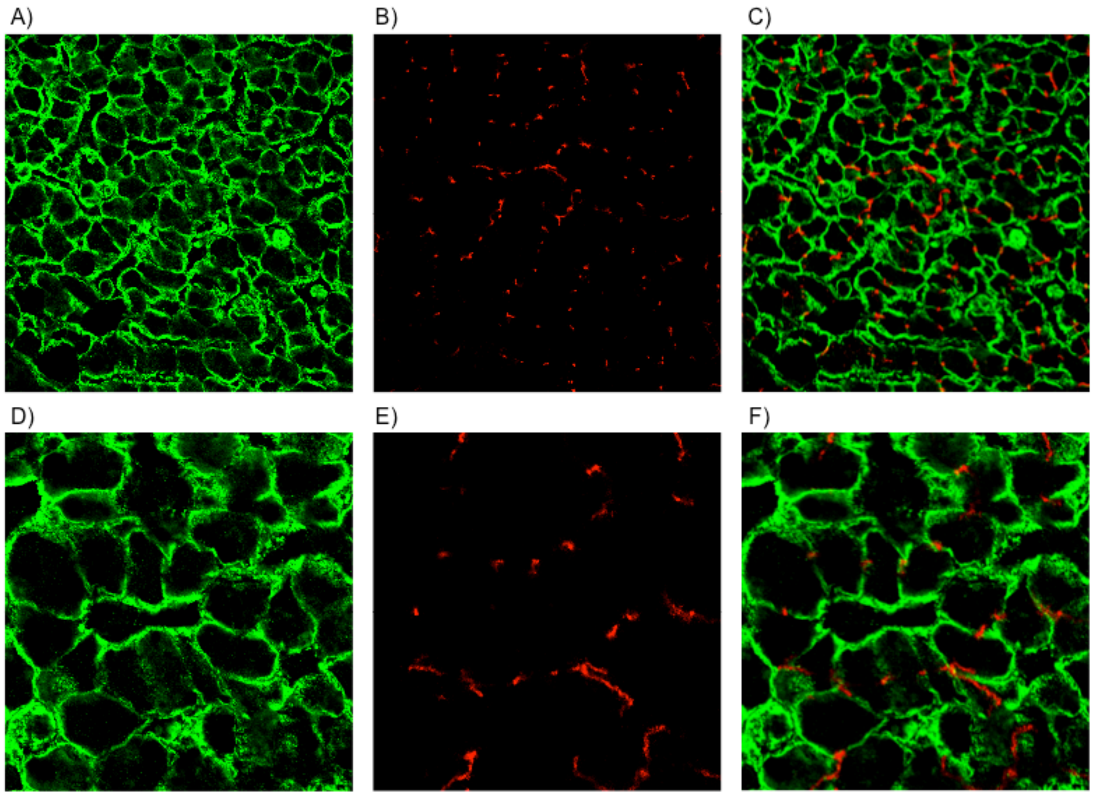


Figure 32. Presence of β_3 GABA$_A$ receptor subunit in liver tissue. (**A-D**) Immunostaining for β_3 GABA$_A$ receptor subunit is shown in green. **(B-E)** the apical marker MRP2 is shown in red. The image pairs A-B and D-F are superimposed in **(C)** and **(F)**. The experiment was carried out three additional times with similar results.

4.3.3 Electrophysiology: patch clamp technique.

We tried to apply the patch clamp technique to IHH cells with the aim to determine the functional properties of the expressed $GABA_A$ receptors. Measurements was extremely difficult due to the tendency of the IHH cells to swell. Unfortunately the promised human primary hepatocytes did not became available for electrophysiological analysis. Exposure of IHH cells to 100 µM GABA did often not result in any currents. **Figure 33** shows an exception with an outword current of about 80 nA.

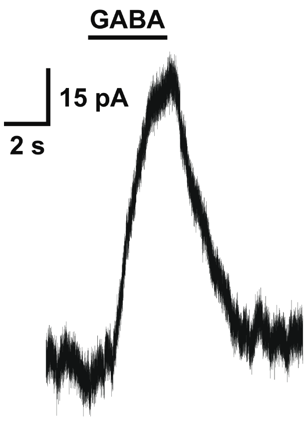


Figure 33. Current measurement in whole cell mode in 100% confluent IHH cells held at 0 mV during treatment with GABA (100 µM). The intracellular solution contained 15 mM Cl^-; extracellular solution contained 155 mM Cl^-. $GABA_A$ receptor was activated by bath application of 100 µM GABA. An outward current of about 80pA was elicited.

4.4 Discussion

Some observations prompted us to study the role of $GABA_A$ receptors in liver. Minuk et al. (2007) described that GABA inhibits the proliferation of the HepG2 human hepatoblastoma cell line and expression of β_3 and ε $GABA_A$ receptor subunits in hepatocytes (Minuk et al., 2007). β_3 (mRNA and protein) was found to be downregulated in liver carcinoma (Minuk et al., 2007). Carcinoma cell lines transfected with β_3 were found to be less aggressive in vivo than non transfected cells (Minuk et al., 2007). β_3 subunit down-regulation was also paralleled by a decrease of the hepatocyte membrane potential and an increase of the cell proliferation. Later studies have shown that

GABA inhibited human liver cancer cell migration and invasion via the ionotropic $GABA_A$ receptor (treatment with $GABA_A$ receptor agonists (T101), but not with $GABA_B$ receptor agonists (Baclofen) showed the specific action through the $GABA_A$ receptor) as a result of the induction of liver cancer cell cytoskeletal reorganization (Chen et al., 2012). Pre-treatment with GABA also significantly reduced intrahepatic liver metastasis and primary tumour formation in vivo (Chen et al., 2012). However, the precise role of GABA and its receptors in human liver cancer cell migration and invasion is not well understood. Specifically, no $GABA_A$ receptor activity had ever been shown.

We chose the immortalized human hepatocyte (IHH) as a model system for further studies. There is evidence that the plasma cell membrane potential influences cell proliferation (Morokuma et al., 2008; Sundelacruz et al., 2009) Depolarization of embryonic cells by mis-expression of a certain type of potassium channels (KCNE1) induced melanocytes to over-proliferate, spread out, and become highly invasive of blood vessels, liver, gut, and neural tube. (Morokuma et al., 2008). Factors, such as neurotransmitters, that change the membrane potential contribute to the control of cell proliferation (Martins and Pearson 2008). The neurotransmitters GABA, has been shown to regulate proliferation of several cell types apart of liver cancer cells, including embryonic stem cells (Andäng et al., 2008), immune cells, (Jin et al., 2013, Bjurstom et al., 2008).

mRNA and protein expression in IHH cells. The mRNA profiles of $GABA_A$ receptor subunits β_3 and ε were investigated with different degrees of confluency in IHH cells (40%, 70%, 90% and 100%). Neither β_3 nor ε mRNA showed significant differences when the cells were 40% or 100% confluent. In vitro studies showed that β_3 can form a functional homomeric receptor upon expression in canine kidney cells (MDCK) (Connolly et al., 1996). Studies in oocytes showed the formation of functional receptor from ε, β_2 and α_1 $GABA_A$ receptor subunits (Bollan et al., 2008). We need additional studies to investigate the possible subunit combination expressed in hepatocytes. At the protein level, we found an inverse correlation between β_3 protein expression and degree of proliferation. As this correlation was observed at the protein level but not the mRNA level our findings suggest a post-transcriptional regulation. Our results are in line with earlier findings (Minuk et al., 2007).

Subcellular localization of β_3 subunit. We investigated the localization of β_3 GABA receptor subunit in healthy liver tissue and found a basolateral localization for the β_3 subunit protein. The apical surfaces in liver cells are exposed to bile cannaliculi and basolateral surfaces are in contact with the blood (**Figure 34**).

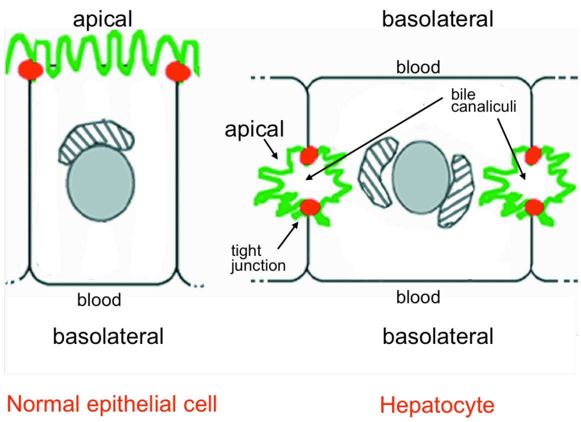


Figure 34. Cell polarization in normal epithelial cells and hepatocytes.

Adjacent epithelial cells are connected by tight junctions. This organization permits regulated import of substances into the body as well as export of substances from the body (Duffield et al, 2008; Mellman and Nelson, 2008; Martin-Belmonte and Mostov, 2008; Müller and Bossinger, 2003). Similarly neurons show a polarized morphology in which they usually have a single axon and multiple dendrites originating from the cell body. The axon (apical) from one cell releases the neurotransmitter which will bind a receptor located on the dendrites (basolateral) of a different cell at the synaptic cleft. The polarity of the neuron thus facilitates the directional flow of information, which is required for communication between neurons and effector cells.

A study in MDCK cells showed specific basolateral targeting of both $\alpha_1\beta_2$ and $\alpha_1\beta_3$ $GABA_A$ receptors. Interestingly, delivery of receptors containing the β_3 subunit to the basolateral domain occurs via the apical surface. These results showed that beta subunits can selectively target $GABA_A$ receptors to distinct cellular locations (Connolly et al., 1996). $GABA_A$ receptors localized in the basolateral membrane of hepatocytes would bind the GABA neurotransmitter in the plasma. It has been show that the GABA concentration in the blood is around 100 nM (Petty, 1994). Interestingly, the betaine GABA transporter (BGT1) (protein and mRNA) and the GABA transporter type 2 (GAT2) were found to be expressed in rat hepatocytes and targeted to the sinusoidal plasma membranes facing blood plasma (Zhou et al., 2012). Therefore hepatocytes are equipped to take up GABA from the plasma and a use for activation GABA receptors in the basolateral membrane cannot be excluded.

Electrophysiology. To investigate whether Isubunit express a functional $GABA_A$ receptor, we conducted electrophysiological studies. As mentioned above the studies were technically difficult, unfortunately we were not able to obtain reproducible results. One of the main problems was the swelling of the cells after clamping. Swelling is a well studied characteristic of the hepatocytes, which is correlated with the presence of chloride channels. $GABA_A$ receptors form also a chloride channels. It cannot be excluded that $GABA_A$ receptor is involved in controlling the swelling. As IHH cells are not polarized it is possible that no functional $GABA_A$ receptors are expressed. The few times we obtained primary hepatocytes for electrophysiological recording, their quality did not allow electrophysiological experiments.

4.5 Outlook

Localization. It will be interesting to localize β_3 subunit in liver cancer tissue and IHH cells. To localize the β_3 subunit in IHH cells a collagen sandwich cell culture model could be established allowing three-dimensional differentiation (Tuschl et al 2009). Furthermore, after the interesting observation of the presence of the BGT1 and GAT2 GABA transporters in the liver we need to understand if these transporters are really involved in GABA transfer from the blood to the hepatocytes and if it regulates the expression of $GABA_A$ receptors in the membrane in physiological or pathological condition such as cancer, where β3 subunit is known to be down-regulated (Minuk et all, 2007), or if, for example BGT1 is only involved in the Betaine transport as previously described (Zhou Y, et al; 2011). It will be interesting to see if also the BGT1 and GAT2 protein expression is varying with the degree of confluency in IHH cells. In addition, it could be studied if $GABA_A$ receptor expression is affected in the liver of mice lacking of the BGT1 protein

Function. The most important aim of my project was to look for $GABA_A$ receptor function. Unfortunately, nobody has been able so far to achieve this. Electrophysiological studies with other hepatocyte cell lines or native hepatocytes may overcome the technical limitations we observed using IHH cells.

Hepatocyte proliferation. Western Blot analysis of the β_3 subunits showed a 5-fold increase in β_3 protein level with increasing confluency of the cells. This suggests that hepatocytes might only express $GABA_A$ receptors when "sensing" other hepatocytes and increase production of the receptors as confluency increases. Additional studies with siRNA directed against β_3 and ε $GABA_A$ receptor subunits will lead to the better understanding of the role of $GABA_A$ receptor in proliferation of the hepatocytes. siRNA approches have successfully been used in hepatocyte cell lines (Shan et al., 2004)., When the hepatocytes start to be connected with each other, a swelling is observed, due to a Cl^- conductance. It will be interesting to understand if GABA is also involved in controlling the swelling or the osmolarity of the cells to optimize the contact with other cells.

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Declaration of Originality

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I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

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I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date 06. 11. 2014

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