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DISSERTATION

Titel der Dissertation

VALERENIC ACID, ACTEIN AND DERIVATIVES: SUBUNIT-DEPENDENT MODULATION OF $GABA_A$ RECEPTORS

Verfasserin

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Table of Contents

1. INTRODUCTION / STATE OF RESEARCH

1.1. BASIS OF NEURONAL EXCITABILITY

At the end of the 18th century Galvani and Volta suggested that propagation of nerve impulses and muscle contraction are based on electrical signals. In 1902, Bernstein hypothesized that in nerve and muscle cells the unequal distribution of potassium (K^+)- ions over the cell membrane and the selective K^+-permeability produces the resting potential and that a transient breakdown of selective permeability induces excitation, allowing other ions to pass the membrane. These changes in ion permeability along the axon of a neuron were suggested to build the backbone of the propagation of nerve impulses. Isolation of the squid giant axon and subsequent electrophysiological experiments by Hodgkin and Huxley in 1952 revealed that ion movements are the basis for the propagation of nerve impulses and local changes of the membrane potential (Zubay, 2000).

In the central nervous system (CNS) communication between neurons is based on both electrical and chemical means. A prerequisite for propagation of electrical signals are gap junctions and a cytoplasmatic continuum between presynapse and postsynapse. A communication based on chemical means (e.g. neurotransmitters or hormones) involves a ligand that travels the distance between the cell by diffusion and binds to specific proteins (Luddens and Korpi, 1995; Rang et al., 2003).

1.2 γ-AMINOBUTYRIC ACID (GABA)

GABA (see structural formula in Fig. 1) is the major inhibitory neurotransmitter in the CNS of the mammals (Sieghart, 1995; Barnard et al., 1998; Sieghart, 2006). GABA is synthesized from glutamate catalysed by two isoforms of the glutamic acid decarboxylase (GAD) GAD 65 and GAD 67 (Erlander et al., 1991). GABA is stored in synaptic vesicles by vesicular neurotransmitter transporters and released from nerve terminals by calcium-dependent exocytosis (Owens and Kriegstein, 2002). There is also evidence for non-vesicular secretion of GABA by e.g. reverse transporter action, a mechanism that might be of particular relevance during brain development (Attwell et al., 1993; Taylor and Gordon-Weeks, 1991). GABA interacts with ionotropic GABA type A ($GABA_A$) and metabotropic GABA type B ($GABA_B$) receptors, which can be localized either pre- or postsynaptic (Bormann, 2000; Owens and Kriegstein, 2002). Reuptake of the neurotransmitter into nerve terminals and/or surrounding glial cells by the plasma-membrane bound GABA transporter (GAT) terminates the action of the neurotransmitter (Cherubini and Conti, 2001). GABA is finally metabolised by a

transamination reaction, which is catalysed by the GABA transaminase (GABA-T) (Owens and Kriegstein, 2002).

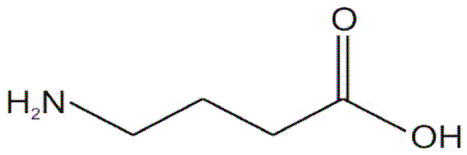


Figure 1 Structural formula of GABA

1.2.1 GABAERGIC NEURONS

GABA is present in approximately 20-50% of all cerebral cortex synapses (Halasy and Somogyi, 1993; Bloom and Iversen, 1971; Hevers and Lüddens, 1998). In the neocortex, most of the GABA-containing neurons are apparently interneurons with few dendritic spines. They are classified as sparsely spiny, aspiny or smooth cells. These cells are further subdivided into groups such as basket cells, chandelier cells, double bouquet cells, local plexus neurons or neurogliaform cells. GABAergic neurons differ in respect to their morphology, neurochemical composition, somatic location and terminal arborization. GABA containing neurons are apparently distributed throughout the cortical lamina (Owens and Kriegstein, 2002).

GABA interneurons are further classified in respect with their intrinsic membrane properties and synaptic connectivity (Connors and Gutnick, 1990; Gupta et al., 2000). Most GABA-containing neurons in the neocortex are generated and migrate from subcortical rather than from cortical locations (Anderson et al., 1997).

It has been proposed that in addition to the local circuit neurons, direct GABAergic afferents project to the cortex from the basal forebrain and the zona incerta (Owens and Kriegstein, 2002).

1.2.2 GABAERGIC SYNAPSES

Neocortial synapses differ in their ultrastructure and are thus divided into 2 groups: type 1 and type 2 synapses (Gray, 1959; Owens and Kriegstein, 2002). Type 1 synapses are characterized by an asymmetrical membrane density at the synaptic cleft and are assumed to be excitatory, while type 2 synapses exhibit a symmetrical appearance and are considered to be inhibitory. There is evidence that more than 15% of all adult cortical synapses are inhibitory and thus, contain GABA.

GABAergic synapses are present in all neocortical layers, and in particular on cell somata, proximal dendrites, axon initial segments, distal dendrites and dendritic spines (Owens and Kriegstein, 2002).

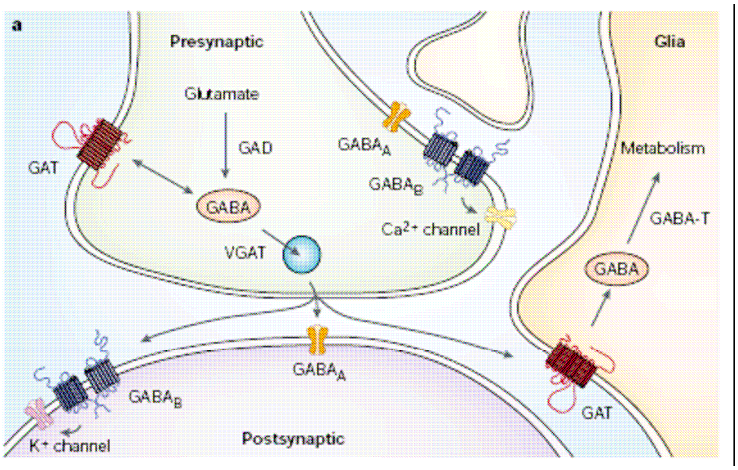


Figure 2 Schematic diagram of the synthesis and transport of GABA at synapses (Owens and Kriegstein, 2002)

GABA mediates its effect by interacting with i.) $GABA_A$ receptors representing ligand gated chloride channels (Sieghart, 1995), ii.) G-Protein coupled $GABA_B$ receptors that modulate Ca^{2+}- and K^+- channels via G-proteins and iii.) $GABA_C$ receptors (Barnard et al., 1998; Bormann, 2000). However, this classification system is controversially discussed (see Barnard et al., 1998; Bormann, 2000).

1.3 GABA TYPE A ($GABA_A$) RECEPTORS

$GABA_A$ receptors, the major inhibitory neurotransmitter receptors, belong to the superfamily of ligand-gated ion channels (LGICs) (Sieghart, 1995; Sieghart, 2006). This family comprises amongst others the nicotinic acetylcholine (nACh receptosr), serotonin subtype 3 (5-HT_3 receptors) or glycine receptors (Barnard et al., 1998; Minier and Sigel, 2004; Conolly and Wafford, 2004).

Ligand-gated ion channels such as $GABA_A$ receptors are commonly formed by assembly of 5 subunits (Conolly and Wafford, 2004, Sieghart, 2006). So far, nineteen different isoforms of mammalian $GABA_A$ receptor subunits have been cloned: $\alpha_{1–6}$, $\beta_{1–3}$, $\gamma_{1–3}$, δ, ε, π, ρ_{1-3} and θ (Barnard et al., 1998; Simon et al., 2004).

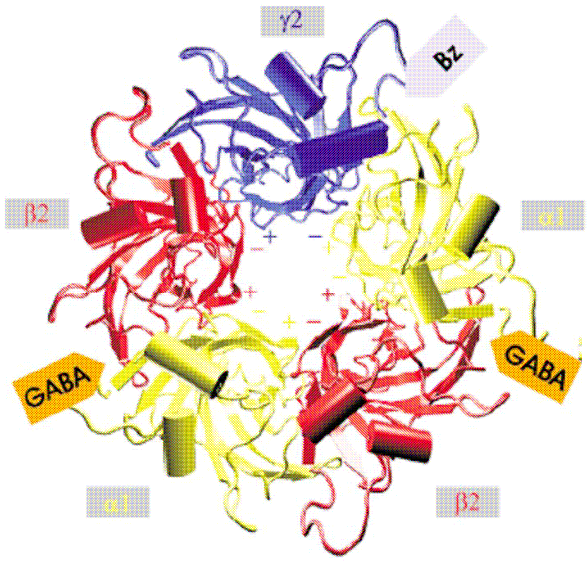


Figure 3 Model structure of the extracellular domains of $GABA_A$ receptor extracellular domains showing the arrangement for α_1, β_2 and γ_2 containing $GABA_A$ receptors (view from extracellular) (Ernst et al., 2003).

The knowledge about the assembly of a certain subunit combination in neurons is limited and many different subunit combinations are expected to be present in the brain (McKernan and Whiting, 1996). There is evidence that the major receptor subtype of the $GABA_A$ receptor in adults comprises α_1-, β_2-, and γ_2-subunits in a 2:2:1 stoichiometry (Chang et al., 1996, Tretter et al., 1997; Baumann et al., 2002, Sieghart and Sperk, 2002).

The putative membrane topology of a single subunit is schematically represented in Fig. 4. Each subunit has an extracellular, β-rich, N-terminal domain of approximately 200 amino acids, which comprises ligand binding sites such as for GABA or benzodiazepines and the highly conserved Cys-loop. 2 β-sheets form a “sandwich” (Brejc et al. 2001) designated as “inner” and “outer” sheet (Unwin et al., 2002). The inner and outer sheet are connected by the signature disulfide bridge (Ernst et al., 2005). The C-terminus is similar sized with 4 hydrophobic membrane spanning domains (M1-M4) (Horenstein et al., 2001). The M1-M4 segments are assumed to be α-helical (Ernst et al., 2005). Because both the N-terminus and C-terminus of $GABA_A$ receptor subunits extend outside the cell membrane, the intracellular M3-M4 loop becomes the most important domain interacting with the intracellular environment, e.g. providing protein-protein interactive domains involved in regulating synaptic localization and intracellular trafficking (Chen and Olsen, 2007). The chloride-conducting pore is flanked by the M2 segments of the 5 subunits (Horenstein et al., 2001).

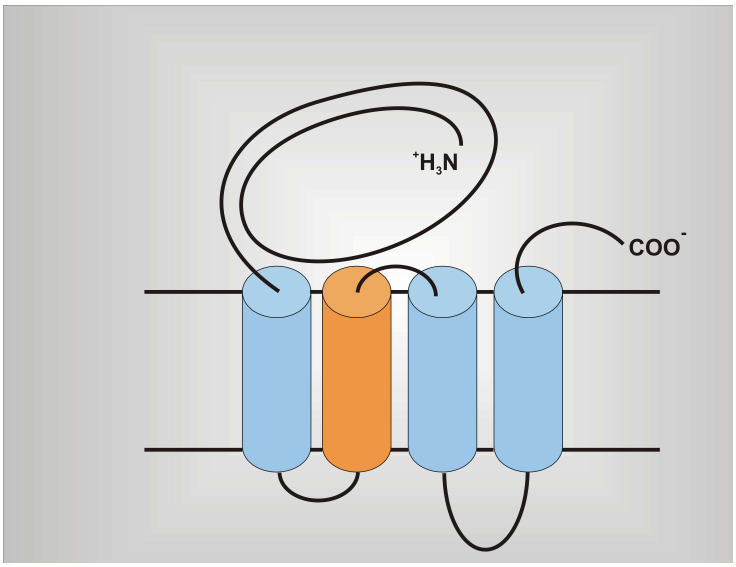


Figure 4 Schematic illustration of a $GABA_A$ receptor subunit

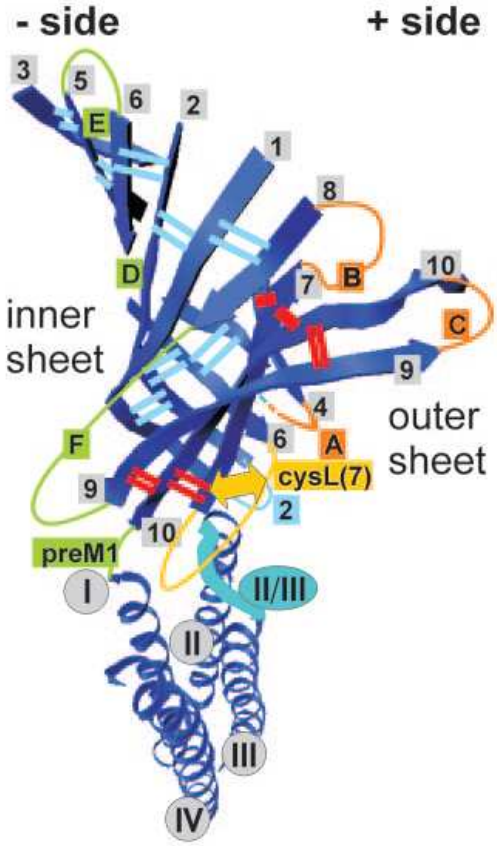


Figure 5

Illustration of the topology of a subunit of cys-loop receptors. The secondary structure motifs that are assumed be conserved are shown in ribbon representation, strand numbering is shown in Arabic numerals, whereas numbering of the membrane spanning helices is shown in Roman numerals. The important topologically conserved but structurally variable regions are indicated only schematically. The hydrogen bonds of the inner sheet are illustrated in light blue and outer sheets' hydrogen bonds are depicted in red. All features associated with the plus side are depicted in orange, those belonging to the minus side are green. The cys-loop is shown in yellow, and the disulfide bond is shown as a yellow double arrow (Ernst et al., 2005).

Another common feature of $GABA_A$ receptors is a highly conserved sequence in the M2 region encompassing the amino acids TTVLTMTT (Korpi et al., 2002).

Upon activation of $GABA_A$ receptors, the intrinsic chloride channel opens. Depending on the reversal potential of the permeating ions (chloride and bicarbonate) the postsynaptic GABA response can be either excitatory or inhibitory. Since the intracellular concentration of chloride in most mature neurons is low, the chloride reversal potential is negative to the action potential threshold. Activation of $GABA_A$ receptors exerts an inhibitory influence on the neuron (Akk et al., 2007). However, there is recent evidence that GABA-mediated responses can be also excitatory especially in immature neurons (Bernard et al., 1998; Ben-Ari, 2002).

An inhibitory postsynaptic potential is triggered by the release of presynaptic GABA that binds to postsynaptic $GABA_A$ channels. IPSCs are characterized by a fast activating current with a rise time of approximately 1 millisecond or less that decays to the baseline over tens or hundreds of milliseconds (Macdonald et al., 2004).

1.4 $GABA_A$ RECEPTOR DYNAMICS

1.4.1 ASSEMBLY OF $GABA_A$ RECEPTORS

Assembly of $GABA_A$ receptor subunits to functional receptors requires specific signaling sequences for the interaction of the subunits (Korpi et al., 2002).

A glia-derived protein binding acetylcholine was identified in the molluscan CNS (Smit et al., 2001). This protein exhibits a high degree of sequence similarity to the N-terminus of nicotinic acetylcholine receptor subunits including the domains that are assumed to form the agonist binding sites and a cysteine loop with 12 intervening amino acids (instead of 13 as reported for e.g $GABA_A$ receptors, see Ernst et al., 2005). It lacks, however, the membrane spanning domains and it forms soluble (i.e. non-membrane-bound), homopentameric complexes (Brejc et al., 2001). In analogy with the acetylcholine binding protein it is assumed that the extracellular N-terminus apparently represents a major determinant for the subunit assembly in ligand gated ion channels (Korpi et al., 2002). A 70 amino acid stretch in the second half of the N-terminal extracellular domain determines homooligomeric assembly of the $GABA_C$ receptor composed of ρ_1 subunits (Enz and Cutting, 1999).

Klausberger et al. (2001a) suggested that the regions α_1(80- 100) and γ_2(91–104) participate in subunit interaction, assembly and formation of the benzodiazepine binding site in recombinant $\alpha_1\beta_3\gamma_2$ receptors. An adjacent region of the γ_2 subunit γ_2(83–90) was suggested to mediate assembly with α_1 and β_3 subunits- (Klausberger et al., 2000).

Truncated N-terminal extracellular domains of $GABA_A$ receptor γ_2 (1-234) and α_1 subunit dimers can form a benzodiazepine binding site, while [^{3}H]muscimol binding apparenly requires the presence of transmembrane domains of both α and β subunits (Klausberger et al., 2001b).

1.4.2 TARGETING AND REGULATION OF MEMBRANE EXPRESSION

De novo synthesis and assembly of $GABA_A$ receptor subunits occurs in the endoplasmatic reticulum. After correct assembly $GABA_A$ receptors are targeted to the plasma membrane via the Golgi apparatus where they might aggregate and anchor to subsynaptic elements to form synaptic clusters. Unassembled receptor subunits are degraded (Kneussel, 2002).

A protein - $GABA_A$ receptor-associated protein (GABARAP)- involved in $GABA_A$ receptor plasma membrane targeting has been identified (Wang et al., 1999). GABARAP comprises 117 amino acids and is expressed ubiquitously in all tissues including several areas of the CNS. The N-terminal domain of GABARAP (amino acids 1–36) is highly charged and features a tubulin binding motif that interacts with both soluble tubulin and polymerized microtubules, while the $GABA_A$ receptor γ_2-subunit interacts with residues 36–68 (Wang et al., 1999; Kneussel, 2002). Residues 37–117 of GABARAP were shown to interact with the tubulin-binding protein gephyrin. Interestingly, this interaction with $GABA_A$ receptors is limited to γ_1, γ_{2S}, and γ_{2L}, but does not occur with other $GABA_A$ receptor subunits (Wang et al., 1999; Nymann-Andersen et al., 2002; Chen and Olsen, 2007).

Chen et al. (2000) suggested that co-expression of GABARAP and different $GABA_A$ receptor subunits leads to $GABA_A$ receptor aggregates that influence the function of surface $GABA_A$ receptors. This indicates an involvement of GABARAP in the organization of GABAergic synapses or alternatively a participation of GABARAP in membrane fusion events underlying organizational processes at GABAergic synapses (Kneussel, 2002). Postsynaptic receptors are likely to be saturated by presynaptically released transmitters. Thus, an increased number of active postsynaptic receptors represents an efficient way of strengthening synaptic efficacy. Processes facilitating the insertion of newly synthesized or recycled receptors into neuronal membranes to maintain a stable cell surface receptor number, therefore seem to be a critical requirement for the regulation of synaptic strength (Kneussel, 2002).

The insulin receptor tyrosine kinase (Wan et al., 1997) and the ubiquitin-like protein Plic-1 (Bedford et al., 2001) have been suggested to be involved in regulation of $GABA_A$ receptor surface expression (Kneussel, 2002).

Insulin causes a rapid translocation of $GABA_A$ receptors from the intracellular compartment to the plasma membrane in heterologous expression systems. This process has been shown to be specifically dependent on the presence of a $GABA_A$ receptor β_2-subunit (Kneussel, 2002).
In neurons, the expression of $GABA_A$ receptors on dendritic and postsynaptic membranes is also enhanced by insulin suggesting that a rapid recruitment of functional receptors is regulated via pathways that include the activation of growth factor receptors (Kneussel, 2002).
However, the receptor density on the membrane is also regulated by negative modulators of the protein degradation machinery at the proteasome. In proteasomes, poly-ubiquitinated substrates are unfolded and degraded to small peptides. In humans, the proteins Plic-1 and Plic-2 represent apparently ubiquitin-like proteins that physically interfere with both proteasomes and ubiquitin-ligases. Thus, overexpression of Plic proteins interferes with the *in vivo* degradation of ubiquitin-dependent proteasome substrates. Plic-1 has been further shown to bind to both α and β subunits of $GABA_A$ receptors leading to an increased number of receptors available for insertion into the plasma membrane. Further support for this scenario comes from the finding that a blockade of the interaction of Plic-1 with $GABA_A$ receptors results in a time-dependent reduction in whole-cell GABA-activated current amplitudes.
Thus, Plic-1 is probably involved in controlling $GABA_A$ receptors subunit half-life (Kneussel, 2002). In contrast, the neurotrophic factor BDNF (Brain-derived neurotrophic) factor has been suggested to negatively modulate $GABA_A$ receptor surface expression (Brunig et al., 2001). A close connection between $GABA_A$ receptor down-regulation and voltage-gated calcium channels has been proposed (Lyons et al., 2001), which is supported by the finding that nifedipine inhibits both GABA-induced increases in calcium concentrations and $GABA_A$ receptor downregulation. Taken together, the surface expression of $GABA_A$ receptors is a regulated by processes including positive and negative modulatory pathways (Kneussel, 2002).

1.4.3 CLUSTERING OF $GABA_A$ RECEPTORS AT SYNAPSES

Gephyrin was initially described as 93 kDa protein that was co-purified with glycine receptors (Pfeiffer et al., 1982). Gephyrin, however, shows a wider expression over the CNS as well as in peripheral tissues (Prior et al., 1992). Gephyrin is a multifunctional protein accounting for both molybdenum cofactor synthesis and the clustering of glycine and $GABA_A$ receptors at inhibitory synapses.

The underlying mechanism of clustering at the synapse, however, is complex and currently not well understood. Removal of gephyrin by gene targeting or mRNA expression interference strongly affects $GABA_A$ receptor clustering (Fritschy et al., 2008).

Mice lacking gephyrin die after birth and exhibit reduced clustering of glycine receptors at their synapses (Feng et al., 1998) and a reduction in $GABA_A$ receptor α_2 and γ_2 subunits in the spinal cord (Kneussel et al., 1999). In primary hippocampal neuronal cultures, diminished synaptically clustered $GABA_A$ receptors have been detected, while the intracellular pool of $GABA_A$ receptors was increased (Korpi et al., 2002). In analogy to the gephyrin-knockout mice, γ_2- knockout mice show also decreased $GABA_A$ receptor clustering (Craig et al., 1996; Essrich et al., 1998), providing evidence for a dominant role for gephyrin and γ-subunits in $GABA_A$ receptor clustering. As for GABARAP, the gephyrin interaction with $GABA_A$ receptors is limited to γ_1, γ_{2S}, and γ_{2L}, (Wang et al., 1999; Nymann-Andersen et al., 2002; Chen and Olsen, 2007).

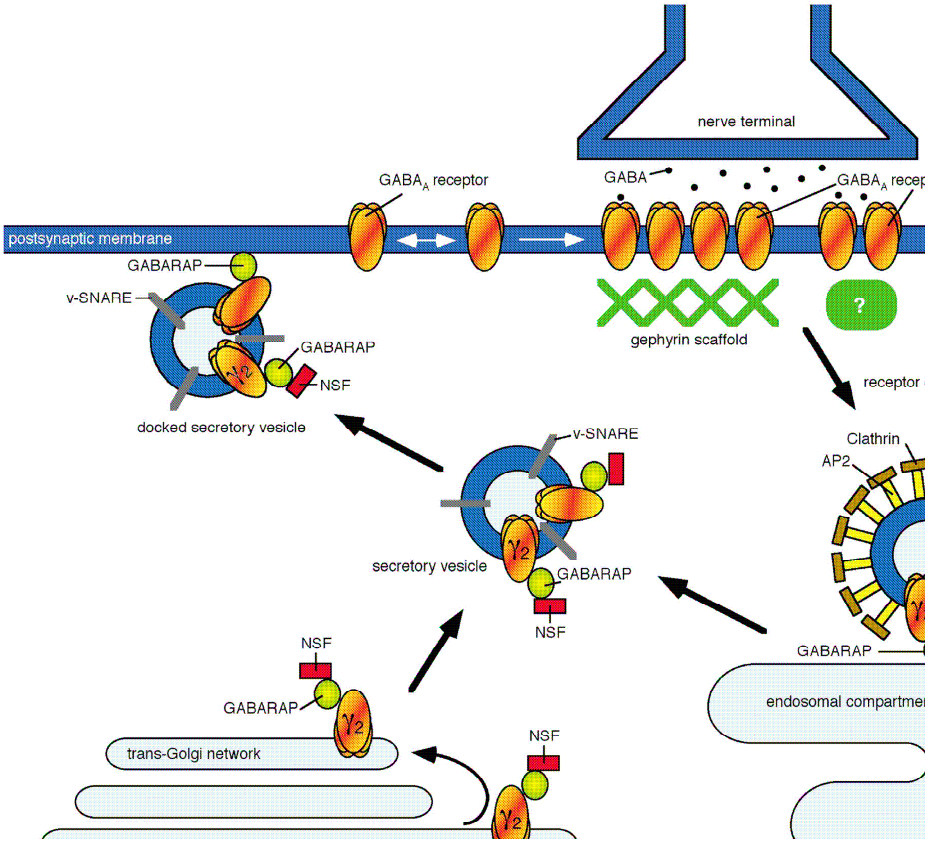


Figure 6 Illustration of $GABA_A$ receptor dynamics (Kneussel, 2002)

1.4.4 $GABA_A$ RECEPTOR INTERNALIZATION AND RECYCLING

The uptake (endocytosis) of extracellular material and membrane proteins into cells via membrane-bound vesicles is mediated by proteins that cycle between the synaptic membrane and intracellular sites (Liang and Huganir, 2001; Marsh and McMahon; 1999; Noel et al., 1999; Kneussel, 2002). There is evidence that $GABA_A$ receptors are internalized from cell bodies and dendrites via clathrin-mediated endocytosis (Kittler et al., 2001; Korpi et al., 2002).

This process (interaction of the adaptin complex AP2 with the $GABA_A$ receptor β and γ subunits) has been shown to be critical for the recruitment of integral membrane proteins into clathrin-coated pits. Blocking the clathrin-dependent endocytosis results in large, sustained increases in the amplitude of miniature IPSCs in cultured neurons suggesting a constant cycle of $GABA_A$ receptors between synaptic sites and endocytic structures (Kneussel, 2002).

1.5 STRUCTURAL DETERMINANTS OF THE AGONIST BINDING SITE

Nicotinic ACh receptor models have strongly influenced the design of structural models for the family of ligand-gated ion channels. The agonist binding site is formed by 6 loops designated A through F (Corringer et al., 2000; Sedelnikova et al., 2005).

GABA binding is assumed to occur at the interface α/β (see Figure 6), whereas benzodiazepines binding site is formed by the α and the γ subunit (Sieghart, 2006).

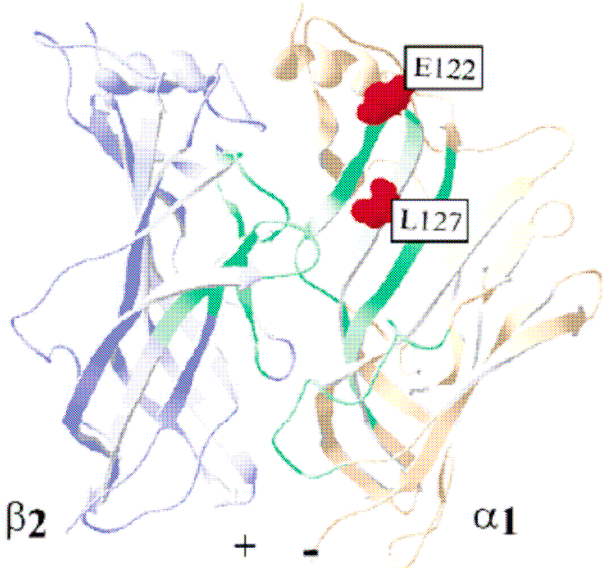


Figure 7

Side view of the GABA binding site at the α/β subunit interface. The putative GABA-binding region is depicted in green (Muroi et al., 2006).

In analogy to the agonist binding site of nACh receptors (see Corringer et al., 2000), the GABA binding site at the α/β interface is predicted to be constituted by 6 loops designated A-

F with several residues of these loops facing the binding site (Boileau et al., 2002a; Ernst et al., 2003; Ernst et al., 2005). α_1 contributes to the loops D and E, whereas loops A, B, C and F are formed by the principal binding site of β_2. Loop A defines approximately the region β_2Trp92-Asp101. This region is assumed to be a β-strand. Leu 99 and Tyr 97 have been identified to line the GABA binding site (Boileau et al., 2002a). Loop B comprises the region β_2Ile154-Asp163. Loop B is formed by β-strand 9, loop 10 and the beginning of β-strand 10. β_2Thr160 and β_2 Asp163 are assumed to line the GABA binding site. Interestingly, mutation E155C produces spontanously open channels by altering both channel gating and agonist binding (Newell et al., 2004). Loop C is formed by the region β_2 V199-S209, residues S204, Y205, R207 and S209 have been shown to face the agonist binding site. In contrast, F200, S201, T202 and G203 do not directly face the binding site, but if mutated they drastically affect the sensitivity to GABA. Loop C has an extended conformation that may traverse the GABA binding site from its rim to its depth. This region, which does not show a regular secondary structure, is assumed to represent an extended coil or loop (Wagner and Czajkowski, 2001). After agonist binding loop C is predicted to move inward towards loop E (Unwin et al., 2002; Celie et al., 2004, Law et al., 2005).

The region of α_1Y59 to K70 comprises loop D. Residues F64, R66 and S68 are major determinants of GABA binding. This region is likely to be constituted by a β-strand (Boileau et al. 1999). Loop E is formed by the region α_1Met113-Leu132 and linked to loop A of the benzodiazepine binding site via an 11 amino acid stretch. In this region Asn 115, Leu 117, The 129 and Arg 131 are assumed to line the agonist binding site (Kloda and Czajkowski, 2007).

Loop F - formed by the region α_1Pro174-Asp191- is poorly conserved in $GABA_A$- receptor subunits. Residues V178, V180, D183 are assumed to contribute to the GABA binding site. Loop F is predicted to be a random coil (Newell and Czajkowski, 2003)

1.6 GATING OF $GABA_A$ RECEPTORS

The binding of a neurotransmitter to its binding site is assumed to result in the opening of the ion channel within the transmembrane domain, a process known as activation or ‘gating’. It has been suggested that neurotransmitter binding to the agonist binding site results in a local perturbation of its structure (Grosman & Auerbach, 2001; Dahan et al., 2004; Beene et al., 2004). This structural change is further propagated along the longitudinal axis of the receptor through the extracellular domain as a ‘wave’ of conformational changes (Grosman et al.,

2000), Finally, a physical gate is moved within the membrane spanning domain, resulting in an ion flux through the pore (Keramidas et al., 2006).

"pin-into-socket mechanism"

For the nicotinic ACh receptor, the copuling of the the extracellular domain with the membrane domains has been suggested to be a "pin-into-socket" mechanism based on the structure published by Miyazawa et al. (2003). It was proposed that a single valine residue in the extracellular domain (V261) enables contact with the transmembrane domains serving as a pivot, permitting a rotation of the extracellular domain induced by agonist binding to be transduced to the M2 segments. The resulting rotation of the M2 segments enables thus the pore to expand at its narrowest point (Kash et al., 2004a).

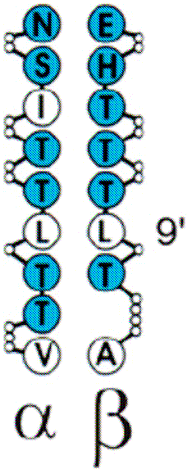
"conformational-wave-mechanism"

Binding of the agonist to its binding site was also alternatively suggested to initiate a series of local movements that are transduced to the membrane spanning domains (Kash et al., 2004a)

In such a scenario, the extracellular N-terminal domain of the $GABA_A$ receptor connected to the M1 domain and thereby interacts with the loop between M2 and M3 (M2-M3 linker). In analogy to a model for the AchBP, it has been suggested that loops 2 and 7 of the $GABA_A$ receptor α subunit interact with a lysine residue in the M2-M3 linker to couple GABA binding to gating (Kash et al., 2003). Moreover, it was proposed that gating induced a conformational change in and/or around the N-terminal half of the M2-M3 linker (Bera et al., 2002). For gating of the ACh receptor it was suggested that the loop 2 of both α subunits is in direct contact with the distal M2 regions (just before the beginning of the M2-M3 linker). Upon acetylcholine binding both loops rotate by 15° around an axis passing through the disulfide bridge. The rotations of the loops are associated with rotations of the M2 domain, which are translated to the gate and might presumably result in the opening of the gate (Unwin et al., 2002; Miyazawa et al., 2003). It was further suggested that the α1K220 residue plays an active role in the transduction process that converts the energy associated with the binding of agonist to the opening of the ion pore. The β-10 strand (pre-M1 segment) appears to be an integral component of a 'coupling zone' that includes loops 2 and 7 and the M2–M3 linker (Keramidas et al., 2006).

1.7 STRUCTURE OF THE ION PERMEATION PATHWAY

The structural basis of ion permeation and selectivity was intensively investigated by Xu and Akabas, 1993; Xu and Akabas, 1996 and Goren et al., 2004. Residues assumed to line the channel are illustrated in Figure 8.



Analysis of the sequence of the M2 segments of different $GABA_A$ receptor subunits reveals a high degree of sequence identity, including several highly or absolutely conserved hydrophilic residues, such as threonine residues at positions 7', 10' and 13', that can be found in all of the $GABA_A$ receptor subunits, while other positions may feature either a serine or threonine (Kash et al., 2004a).

Figure 8 Schematic illustration of the M2 region of α_1 and β_2 subunits, showing the residues contributing to the pore. Residues that can preferentially interact with water molecules are depicted in blue (Kash et al., 2004a).

Moreover, the hydroxylgroups on the side chains are assumed to interact with the water molecules that form the hydration shell surrounding the chloride ions thereby providing energetic stabilization for ions when they enter the pore domain, as previously shown for K^+ channels (Roux and MacKinnon, 1999; Kash et al., 2004a). This mechanism can be applied to both positively and negatively charged ions. A similar hydrophilic sequence (TMTT) was identified in many K^+- channels. In K^+- channels the threonine residues have been shown to comprise the walls of a large water-filled cavity in the center of the channel, deep within the membrane (Doyle et al., 1998; Kash et al., 2004a). Thus, it has been assumed that this hydrophilic region of M2 is presumably not involved in ion selectivity for $GABA_A$ receptors, but it might enhance the channel conductance (Kash et al., 2004a).

1.8 STRUCTURE OF THE SELECTIVITY FILTER

By comparison of the permeability of several inorganic and organic anions, it was suggested that the selectivity filter (narrowest portion of the pore) of $GABA_A$ receptors is 5.6 Å wide (Bormann et al., 1987). Several conserved positively charged residues (arginine and lysine) are clustered around the extracellular end of the M1 and M2 segments. It was suggested that they might play a role in charge selectivity by concentrating negative ions within the channel

vestibule (Kash et al., 2004a). Xu and Akabas (1996) suggested that the selectivity filter might be located at two-thirds of the way down the M2 helix.

There is recent evidence, that the M1–M2 loop of the β subunit plays a pivotal role in determining the ion selectivity of $GABA_A$ receptors (Jensen et al., 2002; Kash et al., 2004a).

1.9 THE GATES

The structure of the resting and desensitized channel gates in the resting or desensitized state in the presence of an agonist, is yet unknown (Kash et al., 2004a). There is evidence, that in some ion channels e.g. in the ClC chloride channel, the selectivity filter simultaneously represents the gate (Furst et al., 2000; Kash et al., 2004a). For K^+- channels it has been shown that gate and selectivity filter are distinct structures located several Å apart (Kash et al., 2004a).

Five 9' leucine residues that are apparently located in the center of the channel-lining M2 segments making a symmetrical contribution have been assumed to form the resting gate of nACh receptors (Unwin, 1995; Brejc et al., 2001). However, there is recent evidence that both the resting gate and the selectivity filter of the nACh receptor are located in the M1-M2 loop (Wilson and Karlin, 1998; Wilson and Karlin, 2001; Kash et al., 2004a).

For $GABA_A$ receptors it was suggested that the top of the channel gate is located in proximity to αV257, which would be approximately two helical turns more cytoplasmatic than the assumed 9´ leucine (Xu and Akabas, 1996; Kash et al., 2004a). Making use of substituted admantane derivatives non-competitive antagonism of different positions within the channel pore of nACh receptors was probed, providing further evidence for the suggestion of the location of the closed gate (Arias et al., 2002; Kash et al., 2004a). More evidence comes from trapping experiments with the open channel blocker picrotoxin inferring that the closed channel gate is located between the picrotoxin binding site (α_1V257) and the channel's extracellular end (Bali and Akabas, 2007)

The structure of the desensitized gate of $GABA_A$ receptors is not known, although it is assumed to be distinct from the resting gate (Scheller and Forman, 2002).

Based on the asymmetric structure of the selectivity filter, both the resting gate and the desensitization gate could represent an asymmetrically "collapsed" or constricted pore as shown e.g. for the selectivity filter of KcsA K^+- channel in the presence of low K^+ concentrations (Zhou and MacKinnon, 2003; Kash et al., 2004a).

1.10 DISTRIBUTION PATTERN OF $GABA_A$ RECEPTOR SUBUNITS

So far, 19 different subunits of mammalian $GABA_A$-receptors have been identified; their expression pattern is heterogeneous. The α_1 subunit represents the most widely expressed subunit among all α-variants. Except in the striatum, the reticular thalamic nucleus and the internal granular layer of the olfactory bulb α_1 subunits are found in all parts of the brain.

Receptors containing α_2 subunits in contrast are found especially in the accessory olfactory bulb, dentate molecular layer, in the hippocampal area CA3, central and lateral amygdaloid nuclei, septum, striatum, accumbens and the hypothalamus. In the olfactory bulb (external plexiform and glomerular layers), the inner layers of the cerebral cortex, the endopiriform nucleus, the amygdala, the lateral septum, the claustrum and the superior colliculus α_3 represents the predominantly expressed α-isoform (Pirker et al., 2000). α_5 subunits are expressed in high density in the external plexiform layer of the olfactory bulb, the endopiriform nucleus and the inner layers of the cerebral cortex. Additionally, α_3- expression was found in the internal granular layer of the olfactory bulb (together with α_2), the subiculum, the Ammon's Horn and in ventromedial hypothalamic nuclei. Furthermore α_5 was detected in the superior colliculus, ventral nuclei of the lateral lemniscus and nuclei of the trapezoid body (Pirker et al., 2000). The diazepam-insensitive α-variants, α_4 and α_6, are predominantly expressed in the thalamus (not including the ventral lateral geniculate, reticular and central medial nuclei), the striatum and nucleus accumbens, the tuberculum olfactorium and the molecular layer of the dentate gyrus (α_4-isoform). α_4 subunits were also detected, even though less dominantly distributed, in the cerebral cortex (mainly outer layers), the hippocampus (CA1), the septum, the colliculus superior (outer layers) and the brainstem.

In granule cells of the cerebellum and the granular layer of the cochlear nuclei especially α_6-comprising $GABA_A$-receptors are expressed. Expression of α_6 in the olfactory bulb and the superior colliculus is still controversially discussed (Pirker et al., 2000).

In contrast to the different α- subunits the β-variants are broadly distributed, in particular in the cerebral cortex. In some areas (mainly subcortical areas and the cerebellum), however, their distribution is complementary. As can be exemplified with high density of β_2- containing receptors in the pallidum and a lower density in the striatum, whereas β_3 represents the main β-variant in the striatum and is found in low concentrations in the pallidum. Strong staining for β_1 is found in the CA2 subfield of the hippocampus. β_2 represents the main β variant in the thalamic nuclei (with exception of the reticular nuclei), moreover it can be found in many

interneurons throughout the brain as well as in the cerebellum (together with β_2). In the hippocampus β_3 is incorporated in most $GABA_A$ receptors (Pirker et al., 2000).

γ_2 represents the predominantly expressed γ- variant. High density of γ_2 was found over the CNS with exception of the thalamus (olfactory bulb, cortex, hippocampus, amygdala, septum and basal forebrain, pallidum, hypothalamus). In contrast, γ_1 subunits shows a very limited distribution pattern. γ_1 was detected in the pallidum, substantia nigra, septum, medial and central amygdaloid nuclei and in the bed nucleus of the stria terminalis. Weak expression of γ_3 mRNA was shown all over the CNS, stronger expression has been reported in cerebral cortex and the medial geniculate nucleus. There is also evidence for γ_3 in the parabrachial nucleus.

δ subunits comprising $GABA_A$ channels are mainly located in the cerebrellar granule cells, the thalamus, the dentate molecular layer and subiculum, cortex and striatum (Pirker et al., 2000; Korpi et al., 2002).

2. MODULATION OF $GABA_A$ RECEPTORS

2.1 BENZODIAZEPINES

Since their introduction in clinics, benzodiazepines (BZs) belong to the most frequently prescribed drugs due to their sedative and anxiolytic properties in the treatment of anxiety disorders, insomnia, muscle spasms and epilepsy (Bateson, 2004; Savic et al., 2008). Benzodiazepines with a short plasma half-live (such as triazolam) are usually administered for the treatment of sleep disorders in order to avoid next day “hangover” effects. Benzodiazepines prescribed for the treatment of anxiety disorders such as clonazepam or lorazepam, however, display a longer half-live (Bateson, 2004).

The use of benzodiazepines, however, is also associated with a number of unwanted side effects such as ataxia, daytime sedation, loss of coordination and impairment of memory and cognition (Kales et al., 1986; Kales et al., 1987). There is evidence that long-term administration of benzodiazepines is associated with the development of tolerance and an increased risk of physical and psychological dependence. After sudden cessation of benzodiazepine administration withdrawal symptoms are observed (Bateson, 2004). The underlying mechanism is still unknown. A simple down-regulation of the total $GABA_A$ receptor number does apparently not occur (Bateson, 2002).

Until the discovery of a high-affinity binding site for benzodiazepines on $GABA_A$ receptors, the molecular target for benzodiazepines remained elusive (Mohler and Okada, 1977; Braestrup and Squirres, 1977; Sigel and Buhr, 1997). Drugs not comprising the classical benzodiazepine structure such as β-carbolines, imidazopyridines, triazolopyridazines or

cyclopyrrolones have been shown to interact with the putative benzodiazepine binding site (Sieghart, 1995).

At synapses, $GABA_A$ receptors are activated by a rapid exposure to high concentrations of GABA. Benzodiazepines apparently prolong the decay of spontaneous miniature postsynaptic currents (mIPSC). It was shown that the amplitude of the mIPSCs is enhanced by benzodiazepines in different neuronal systems (Hajos et al., 2000) suggesting that the benzodiazepine-induced increased GABA affinity results in recruitment of additional $GABA_A$ receptors (Mohler et al., 2001).

It is assumed that the enhancement of inhibitory GABAergic neurotransmission induced by benzodiazepines is based on the prolonged decay of the mIPSC. The increase of mIPSC amplitude is probably not based on an enhancement of the mIPSC peak amplitude, but more likely reflects the summation of several mIPSCs due to the drug-induced prolongation of the current (Mody et al., 1994; Mohler et al., 2001).

2.1.1 THE BENZODIAZEPINE BINDING SITE

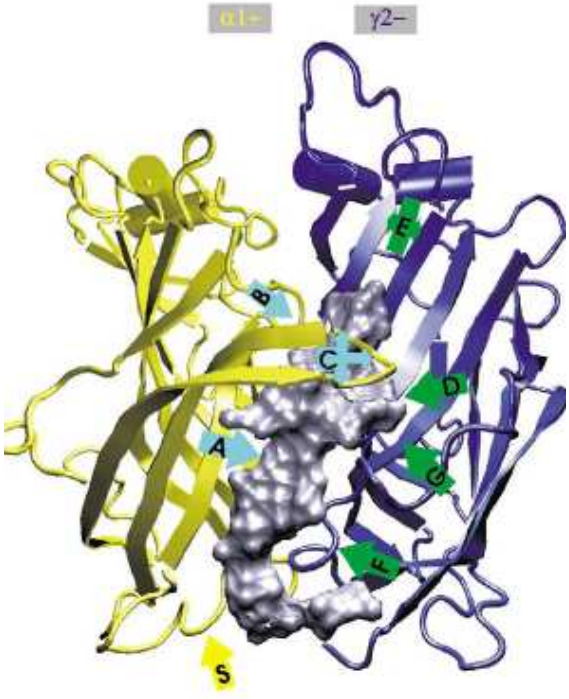


Figure 9 Illustration of the putative benzodiazepine binding pocket. Side view of the α and γ subunits, the membrane would be on the bottom of the figure. (Ernst et al., 2003).

The α_1 subunit containing residue α_1H101 has been identified as the main subunit photoaffinity labelled by [^{3}H] flunitrazepam (Duncalfe et al., 1996). Additionally, a γ_2 subunit is a prerequisite for $GABA_A$ receptor modulation by benzodiazepines (Sigel et al., 1990; Sigel and Buhr, 1997).

In multiple binding studies several amino acid residues have been shown to influence benzodiazepine binding: Mutation of α_3E225G (glycine is the homologous residue in the α_1 subunit) increased the affinity for zolpidem and Cl218872 (Pritchett and Seeburg, 1991). Substitution of H101 by arginine in the α_1 subunit (α_1H101R) resulted in a complete loss of diazepam, zolpidem and Cl218872 binding, whereas the binding affinity of [^{3}H]Ro 15-4513 was not affected (Wieland et al., 1992).

Mutations α_6R100H, α_6P161T, α_6E199G and α_6I210V (mutation to the corresponding residues in the α_1 subunit) drastically changed the affinities of benzodiazepine ligands (Wieland and Luddens, 1994). Mutation α_1Y159S induced a complete loss of [^{3}H]flumazenil binding (Amin et al., 1997). After mutating Tyrosine 209 in the α_1 to glutamine (α_1Y209Q) binding of [^{3}H]flumazenil and [^{3}H]flunitrazepam was abolished, while [^{3}H]muscimol binding was not affected (Buhr et al., 1997c). In contrast, mutation α_1T206A increases the enhancement of I_{GABA} by diazepam and zolpidem (Buhr et al., 1996).

Two amino acids in the γ_2 subunit (γ_2F77 and γ_2M130) confer benzodiazepine sensitivity (Buhr and Sigel, 1997; Buhr et al., 1997a,b; Wingrove et al., 1997). Mutation γ_2F77I leads to a complete loss of zolpidem, Cl218872 and DMCM binding, whereas high-affinity flunitrazepam binding was not affected (Wingrove et al., 1997).

γ_2F77Y apparently increased the affinity for zolpidem and Cl218872 (Buhr et al., 1997b). Substitution of γ_2M130 by leucine results in drastically reduced zolpidem binding (Buhr and Sigel, 1997). I77 and L130 in γ_1 subunits correspond apparently to F77 and M130 in γ_2 subunits (Wingrove et al., 1997).

The ligand-binding sites of neurotransmitter receptors such as nicotinic ACh, glycine and $GABA_A$ receptors are apparently formed by homologous amino acid loops located at the subunit interfaces (Galzi and Changeux, 1995). There is evidence that the GABA binding site and the benzodiazepine binding pocket are homologous. Moreover, not only homologous amino acid loops, but also multiple homologous amino acids are involved in both GABA and benzodiazepine binding. γ_2F77/α64, α159/β157, α206/β202 and α209/β205 are apparently directly homologous to each other, while α200, α211 and γ130 are assumed to be located in

homologous regions to β205 and α120. It has therefore been suggested that many of the amino acid residues in the benzodiazepine binding site are directly homologous to amino acids in the GABA binding site. In analogy to GABA binding that is assumed to occur at α/β subunit interface, the benzodiazepine binding pocket is also assumed to be located at a subunit interface. Interestingly, no homologous amino acid for α_1H101 has been identified in the GABA binding site (Sigel and Buhr, 1997).

Additional amino acids determining benzodiazepine binding affinity include αY159, αY161, αT206, αY209, amino acid residues that are located in different regions of the $GABA_A$ protein, which have been suggested to be located close to each other. It has been proposed that both the α- subunit and the γ-subunit fold back after 50 amino acid residues. The correct three-dimensional arrangement of the benzodiazepine pocket remains to be established. It has been suggested that the region 199-209 of the α-subunit represents a α-helix (Sigel and Buhr, 1997). α_1G200, γ_2A79 and γ_2T81 have been suggested to form part of the benzodiazepine binding pocket (Berezhnoy et al., 2003). The region comprising residues γ_2F77, γ_2A79 and γ_2T81 is assumed to form a β-strand and to undergo a conformational change during the gating process. Flurazepam is in direct contact with γ_2F77 and it might occupy space within the binding site that is close to γ_2A79 (Teissère and Czajkowski, 2001). Although a direct interaction of flurazepam with the alanine residue is unlikely, the small size of alanine might be crucial for providing space for benzodiazepines of different size. Berezhnoy et al. (2003) suggested that γ_2A79 is most probably located in the access pathway of the benzodiazepine ligand to the binding pocket. Moreover, residue γ_2D75 was suggested to play an important role in maintaining the architecture of the benzodiazepine binding pocket (Teissère and Czajkowski, 2001).

An interaction of the 5'-phenyl substituent of classical benzodiazepines with γ_2F77 via π-π stacking was hypothesized by Buhr et al., 1997b and Sigel et al., 1998. Amino acid residue α_1H101 seems to be involved (McKernan et al., 1998; Davies and Dunn, 1998) as α_1H101C covalently interacts with a reactive group attached to the carbon atom in diazepam that normally carries a chloride atom (Berezhnoy et al., 2003).

2.1.2 BENZODIAZEPINE MECHANISM OF ACTION

Binding of benzodiazepines is only the first step in enhancing I_{GABA} (for review, see Colquhoun, 1998). The second step is a conformational change (Boileau and Czajkowski, 1999) coupling benzodiazepine binding to an increased open frequency of $GABA_A$ channels

(Rogers et al., 1994). In contrast to barbiturates or neuroactive steroids benzodiazepines enhance GABA binding and increase the frequency of GABA-gated channel opening (Macdonald and Twyman, 1992; Vicini et al., 1987). They do, however, neither directly gate the receptor nor they alter the mean open time of the channel (Vicini et al., 1987; Campo-Soria et al., 2006).

Although binding occurs at the N-terminus, there are multiple domains of the receptor involved in transducing binding to an enhancement of $GABA_A$ receptor current (Jones-Davies et al., 2005). The classical concept of benzodiazepine enhancement of I_{GABA} is explained by an increase of the apparent affinity of the GABA binding site for the agonist (Campo-Soria et al., 2006). From single channel studies there is evidence, that diazepam increases the rate at which monoliganded receptors open, although actually two GABA molecules are required for channel opening (Twyman et al., 1989; Rogers et al., 1994). It has been suggested that binding of benzodiazepines to the interface of α and γ_2 subunits induces a conformational change that shortens the distance between the two subunits therefore transducing binding to allosteric modulation of $GABA_A$ receptors (Teissère and Czajkowski, 2001). Campo-Soria et al. (2006) suggested that benzodiazepine binding destabilizes the closed state of the receptor and benzodiazepines might act as very weak partial agonists for the $GABA_A$ receptor.

Making use of chimeras (γ_2-δ) and site-directed mutagenesis a crucial role of the distal portion of the M1 region, the distal portion of the M2 region and the M2-3 linker in coupling was identified. The pre-M1 region plays apparently a crucial role in transducing binding to gating. This hypothesis is based on the assmption that the pre-M1 region physically connects both the N-terminus (harbouring the putative binding site) and the M2-region (Jones-Davis et al., 2005).

Further support for such a scenario comes from a $GABA_A$ receptor model by Trudell and Bertaccini (2004) indicating that the N-terminal region of the M1 before the conserved P243 lines the receptor pore by intercalating between M2 channel-lining domains. Three residues γ_2Y235, γ_2F236 and γ_2T281 have been identified in this region to be crucial for transduction (Jones-Davis et al., 2005). Residues γ_2I282 and γ_2T281 in the M2-M3 linker region - that are also involved in formation of an anaesthetic binding site (Jenkins et al., 2001)- have been shown to participate in “benzodiazepine transduction”, suggesting a scenario where the physically adjacent and intercalated extracellular M1 and M2 domains of the receptor act in concert to enhance $GABA_A$ receptor gating (Jones-Davis et al., 2005).

It has been proposed that diazepam induces a conformational change of $GABA_A$ receptors that is structurally distinct from the closed, open, GABA-bound or desensitised states (Williams

and Akabas, 2000). However, Bianchi and Macdonald (2001) reported an enhancement of spontaneous $GABA_A$ receptor currents by benzodiazepines, a finding that does not agree with the classical mechanism of benzodiazepine action by enhancing microscopic affinity of the GABA binding site.

The latter concept suggests that benzodiazepines facilitate gating by a mechanism similar to that of GABA. An analogy between gating models of ACh receptors (Unwin et al., 2002) and $GABA_A$ receptors (Kash et al., 2003) suggests that GABA binding to the interface of α and β subunits initiates a conformational change in the N-terminal region of the β-subunit. This conformational change results in a rotational opening of the girdle of the pore via the M2-M3 linker- a process that requires a certain amount of energy. Structurally, these domains are localized in a manner to promote communication between these domains, as the N-terminal loop 7 is localized in parallel to the M2-M3 linker. Both loops have been shown to be flexible. On agonist binding the N-terminus undergoes a constriction resulting in a movement of the M2-M3 loop to the loop 7 of the N-terminus (Kash et al., 2003; Jones-Davies et al., 2005).

Benzodiazepine binding at the α/γ interface might use an analogous pin-into-socket mechanism, which couples the N-terminal binding pocket (pin) through the M2 and the proximal portion of the M2-M3 loop (socket) (Jones-Davies et al., 2005).

Transduction via the γ2 subunit might reduce the energy required for activation of $GABA_A$ receptor agonist binding sites by supplying a certain amount of energy of its own, that is, however, not sufficient to induce an opening of the channel. Jones-Davies et al. (2005) assumed, that benzodiazepines affect $GABA_A$ receptors by lowering the energy barrier that is required for GABA-mediated gating.

Benzodiazepines produce a biphasic enhancement of $\alpha_1\beta_2\gamma_{2L}$ receptors. The first component in the nanomolar concentration range is dependent on the presence of a γ-subunit and sensitive to antagonism by flumazenil. The low affinity component is mediated via $\alpha_1\beta_2$ receptors that are sensitive to benzodiazepines at micromolar concentrations. Two residues in the M2 and M3 region have been identified to confer sensitivity to the micromolar component of potentiation without affecting the nanomolar component (Walters et al., 2000).

2.1.3 EFFECTS OF BENZODIAZEPINES ARE MEDIATED BY DIFFERENT $GABA_A$ RECEPTOR SUBTYPES

The relevance of $GABA_A$ receptor subtypes in mediating the different effects of benzodiazepines was established based on a gene knock-in strategy. Mice carrying mutations in the benzodiazepine binding pocket (point mutations α_1H101R, α_2H101R, α_3H1261R or

α_5H105R render the receptor insensitive to benzodiazepine modulation) were generated. These mice were expected to fail to display some effects of benzodiazepines and thus, the contribution of a certain subtype to a certain effect of benzodiazepines could be deduced (Rudolph et al., 1999; Rudolph et al., 2001; Mohler et al., 2002).

2.1.3.1 $GABA_A$ RECEPTORS MEDIATING SEDATIVE-HYPNOTIC EFFECTS

Sedative and hypnotic effects by benzodiazepines are mediated by $GABA_A$ receptors containing α_1 subunits. This was demonstrated by the lack of motor activity depressing by diazepam and zolpidem action in the α_1H101R mouse line (Rudolph et al., 1999; Crestani et al., 2000). This finding is supported by McKernan et al. (2000) with α_1-subunit-selective benzodiazepine binding site ligands.

Sedative effects of anaesthetics such as etomidate are also likely to be mediated by β_2 subunit containing receptors. A similar approach was chosen (rendering the β_3 subunit insensitive to modulation of etomidate by the mutation β_3N265M). It was shown that mutant mice recovered their motor abilities quicker than wild type mice after i.p. application of etomidate (Reynolds et al., 2003).

2.1.3.2 $GABA_A$ RECEPTORS MEDIATING AMNESIA

Anterograde amnesia is one of the typically reported side effects of benzodiazepine treatment. The impairment of memory by diazepam is related to α_1 containing receptors (Rudolph et al., 1999; Mohler et al., 2001)

2.1.3.3 $GABA_A$ RECEPTORS MEDIATING ANTICONVULSANT EFFECTS OF BENZODIAZEPINES

The anticonvulsant effect of diazepam is markedly decreased in the α_1H101R mouse line. There is evidence that other than α_1 containing $GABA_A$ receptors are involved in mediating the anticonvulsant effects, as the residual activity in α_1H101R mice could be reversed by flumazenil. Thus, $GABA_A$ receptors containing α_1 subunits are assumed to mediate partially but not elusively the anticonvulsant effects of benzodiazepines (Mohler et al., 2001).

2.1.3.4 $GABA_A$ RECEPTORS MEDIATING ANXIOLYTIC EFFECTS OF BENZODIAZEPINES

There is evidence that anxiolysis after benzodiazepine treatment results from modulation of a population of neurons expressing $GABA_A$ receptors containing α_2 subunits, as the α_2 mutated mouse line is resistant to the anxiolytic effects of benzodiazepines. It was therefore suggested that ligands selectively interacting with α_2-containing $GABA_A$ receptors represent promising leads for future anxiolytic drugs (Low et al., 2000). Moreover, the majority of $GABA_A$ receptors expressed in the amygdala, an area associated with strong impact on anxiety and fear, contain α_2 subunits (LeDoux, 2000; Pirker et al., 2000). However, there is growing evidence, that drugs specifically modulating α_3 containing receptors also exert anxiolytic effects (McKernan et al., 2000). It has been thus suggested that both α_2 and α_3 containing receptors contribute to the anxiolytic effects of benzodiazepines (Atack et al., 2006). The α_2H101R mice failed, however, to display the anxiolytic effects of diazepam. It was suggested that higher doses of diazepam are required to achieve anxiolysis in the mouse line and that the anxiolytic effect might have been obscured by sedation (Morris et al., 2006). An α_3-selective inverse agonist has been reported to be anxiogenic (Collins et al., 2002), whereas an agonist selectively interacting with α_3-comprising receptors has been shown to be anxiolytic *in vivo* (Dias et al., 2005). Another compound with activity on α_2, α_3 and α_5 containing receptors while displaying no activity on α_1 containing receptors has been shown to reduce anxiety in both wild type and α_2H101R mice. These findings underline the role of α_3- containing receptors in anxiolysis. A contribution of α_5-containing receptors to anxiolysis is unlikely (Collinson et al., 2002; Morris et al., 2006).

2.1.3.5 $GABA_A$ RECEPTORS MEDIATING MYORELAXATION

There is evidence that the myorelaxant effects of benzodiazepines are mainly mediated by $GABA_A$ receptors containing α_2 subunits (Crestani et al., 2001), however at high doses α_3 containing receptors are also involved (Mohler et al., 2001).

2.1.3.6 $GABA_A$ RECEPTORS MEDIATING ANALGESIC EFFECTS

A common concomitant phenomenon of inflammatory diseases and neuropathic insults is severe and debilitating pain that might become chronic and that does not respond to analgesic treatment. An impaired synaptic inhibition in the spinal dorsal horn is assumed to contribute essentially to this phenomenon. Enhancement of spinal GABAergic neurotransmission via

enhancement of $GABA_A$ receptors might, thus, be a promising approach. In the spinal dorsal horn presumably $GABA_A$ receptors containing α_2 and/or α_3 containing receptors are expressed. Selectively targeting of these receptors may induce analgesia (Knabl et al., 2008). A benzodiazepine site ligand selectively interacting with $GABA_A$ receptors containing α_2, α_3 or α_5 subunits is highly efficient in relieving inflammatory and neuropathic pain, without exhibiting unwanted sedation, motor impairment and tolerance development (Knabl et al., 2008).

2.1.4 MODULATION OF $GABA_A$ RECEPTORS CONTAINING γ_1 SUBUNITS

Substitution of γ_2 by γ_1 or γ_3 drastically alters the effects of benzodiazepines on $GABA_A$ receptors (Hevers and Luddens, 1998). γ_1 and γ_3 subunits are apparently less abundant in the central nervous system compared to γ_2 (Pirker et al., 2000; Sieghart and Sperk, 2002). The pharmacology of γ_1 and γ_3 containing receptors is, however, less well understood compared to γ_2 containing subtypes. Ymer et al. (1990) reported a reduced affinity for the benzodiazepine antagonist flumazenil and the inverse agonist DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline) in $\alpha_1\beta_1\gamma_1$ receptors. Ro 15-4513, β-CCM and DMCM changed their direction of action and became positive modulators of $\alpha_{1/2/3}\beta_1\gamma_1$ receptors compared to $\alpha_{1/2/3}\beta_1\gamma_2$ receptors. Our group investigated the interaction of 21 ligands of the benzodiazepine binding site with $\alpha_1\beta_2\gamma_1$ receptors. Triazolam, clotiazepam, midazolam, CGS 20265 and CGS 9896 were identified as potent and efficient modulators of $GABA_A$ receptors containing γ_1 subunits (Khom et al., 2006).

2.2 BARBITURATES

After introduction of barbiturates in medical practice in 1934, these drugs interact with multiple targets in the central nervous system. Besides inhibition of excitatory amino acid-gated receptors (Marszalec and Narahasi, 1993) and voltage-gated Ca^{2+} channels (Leslie, 1987) $GABA_A$ receptors represent the major target for hypnotic-anaesthetic barbiturates such as pentobarbital and phenobarbital (Rho et al., 1996). There is evidence that barbiturates exert three different actions on $GABA_A$ receptors: at low concentrations they potentiate agonist-induced currents (Macdonald and Barker, 1979), at higher concentrations they directly activate the receptor in the absence of an agonist (Yang and Olsen, 1987) and at even higher concentrations, barbiturates block $GABA_A$ receptors (Drafts and Fisher, 2006).

On the single channel level, barbiturates increase the mean open time of GABA-activated channels (Macdonald et al., 1989). Interestingly, the kinetic properties of GABA-induced currents in the presence of modulating concentrations of pentobarbital differ from GABA-induced currents in the absence of pentobarbital (Rho et al., 1996).

So far, binding of barbiturates to $GABA_A$ receptors could not be investigated, as barbiturates exhibit a low receptor affinity (Sieghart, 1995). Barbiturates enhance [^{3}H]GABA, [^{3}H]muscimol and [^{3}H]flunitrazepam binding (Olsen, 1982). Moreover, in contrast to convulsants such as picrotoxinin, TBPS, pentamethylenetetrazole that inhibit [^{35}H]TBPS binding competitively, central depressant barbiturates allosterically modulate the [^{35}H]TBPS binding site. Thus, the barbiturate binding site is presumably distinct from the GABA, benzodiazepine or DHP/TBPS binding site (Sieghart, 1995).

The three distinct actions of barbiturates on $GABA_A$ receptors are dependent on the subunit composition (Thompson et al., 1996). Modulation and direct activation are apparently structurally separable (Dalziel et al., 1999; Chang et al., 2003). In general, there is evidence that α and β subunit isoforms influence the modulation of $GABA_A$ receptors by barbiturates (Thompson et al., 1996). Receptors comprising β_2 or β_3 subunits are more efficiently modulated by barbiturates than those containing β_1 subunits (Drafts and Fisher, 2006).

Mutation T262Q in the β_1 subunit abolishes modulation of $GABA_A$ receptors by barbiturates. This, however, does not affect direct activation (Dalziel et al., 1999) as well as mutation β_2G219F eliminates the potentiating effect of pentobarbital (Carlson et al., 2000).

β_3 containing $GABA_A$ receptors seem to contribute to some of the *in vivo* effects of barbiturates. β_3N265M mice showed a strongly reduced duration of the loss of righting reflex and lacked completely the loss of hindlimb withdrawal reflex (Zeller et al., 2007).

2.2.1 DIRECT ACTIVATION OF $GABA_A$ RECEPTORS BY BARBITURATES

Several point mutations in the β_2 subunit (mutation of residues β_2Y157, β_2T160, β_2T202 and β_2Y205) abolished activation of $GABA_A$ receptors by GABA, however did not affect direct activation by barbiturates. This suggests the existence of two distinct sites for activation by GABA and barbiturates (Amin and Weiss, 1993; Rho et al., 1996). Bicuculline and picrotoxin block pentobarbital-induced currents, the latter, however, with higher efficiency. Bicuculline was assumed to inhibit the action of pentobarbital allosterically. Upon rapid termination of barbiturate perfusion of cells expressing $GABA_A$ receptors a transient increase (rebound) in both pentobarbital-induced or pentobarbital-potentiated GABA-induced current was observed,

that could result from a rapid dissociation from a low-affinity channel blocking site facing a relatively slow relaxation of the agonist effect (Rho et al., 1996).

The α-subunit isoform of $GABA_A$ receptors substantially affects direct activation by barbiturates. Pentobarbital gates most efficiently $GABA_A$ receptors comprising α_6 subunits (Thompson et al., 1996). A single amino acid residue located in the N-terminal domain of the α_6 subunit (α_6Thr69) regulates the efficacy of pentobarbital. When mutated to either lysine, histidine or isoleucine this residue drastically reduces the ability of pentobarbital to activate $GABA_A$ receptors directly. There is evidence that residue α_6Thr69 lies at the end of loop D (Drafts and Fisher, 2006). The β subunit is assumed to contribute to the GABA binding site via its "plus side" including loops A and B and the α subunit forms the "minus side" by providing loops D and E (Ernst et al., 2005). Thus, α_6Thr69 is suggested to play an important role in pentobarbital binding or in transducing the binding signal (Drafts and Fisher, 2006).

2.3 GENERAL AND VOLATILE ANAESTHETICS

The ways in which general anaesthetics produce their effects on the CNS are still not completely understood. Historically, general anaesthetics were considered to interfere with membrane fluidity, thereby affecting cellular events. Recent evidence suggests that anaesthetics interact with proteins within the plasma membrane (Franks and Liebs, 1994). Several ion channels are modulated by anaesthetics at clinically relevant concentrations (Krasowski and Harrison, 1999; Thompson and Wafford, 2001). The GABAergic system is considered to be a likely target for multiple anaesthetics (Reynolds et al., 2003). Only ketamine, α_2-adrenergic agonists and xenon target receptors other than $GABA_A$ channels (Franks et al., 1998; Belelli et al., 1999; Miller et al., 2002). In analogy to barbiturates (see chapter 2.2) anaesthetics such as propofol or isoflurane exert three distinct effects on $GABA_A$ receptors: at clinically relevant concentrations they enhance GABA-induced chloride currents, at concentrations higher than those required for potentiation they directly gate $GABA_A$ receptors in the absence of GABA and at still higher concentrations, they block both GABA- and anaesthetic-induced currents (Orser et al., 1994; Belelli et al., 1999; Bali and Akabas, 2004; Hemmings et al., 2005; Rosen et al., 2007). The different actions may also reflect distinct binding sites. Support for this hypothesis comes from the observation that the conformation of segment M3 and the surrounding domains are distinct in the presence of enhancing or directly gating concentrations of propofol (Williams and Akabas, 2002). Direct activation, at least for some anaesthetics, is apparently mediated via a binding site distinct from the GABA binding pocket (Rosen et al., 2007). Single channel studies suggest that

general anaesthetics and GABA induce a similar conductance, however infer different kinetics (Jackson et al., 1982). The open-state structure of the channel seems to be similar in the presence of either general anaesthetics or GABA. Upon activation by GABA residues α_1T261 and β_1T256 are brought in close proximity. This effect is not seen at potentiating concentrations of anaesthetics, but during direct activation induced by anaesthetics (Rosen et al., 2007). There is evidence that the occupancy of these sites stabilises different receptor states or an ensemble of receptor states (Rosen et al., 2007). Single channel studies revealed that- in analogy to barbiturates- general anaesthetics increase the channel mean open time (Twyman and Macdonald, 1992).

Several amino acid residues involved in mediating the effects of both intravenous and volatile anaesthetics have been identified: mutations in the α-subunit affect the effects of volatile anaesthetic, while intravenous anaesthetics are sensitive to mutations in the β-subunit (Belelli et al., 1997; Mihic et al., 1997; Mascia et al., 2000; Krasowski et al., 2001; Nishikawa et al., 2002).

Insights into putative anaesthetic binding sites were provided by studies on mutant receptors or chimeras (Williams and Akabas, 2002; Bali and Akabas, 2004). However, it is almost indistinguishable to judge whether these residues are involved in ligand binding or in transducing the effect (Colquhoun, 1998). Despite these limitations there is evidence that anaesthetics interact with $GABA_A$ receptors in a region close to the extracellular end of membrane spanning domains (Belelli et al., 1999; Krasowski and Harrison, 1999; Miller, 2002). This region has been suggested to be lined by the non-channel lining face of the M2 segment (Xu and Akabas, 1996) and by residues from the M3 as well as from M1 and M4 (Bali and Akabas, 2004).

2.3.1 ETOMIDATE

Etomidate enhances chloride currents through $GABA_A$ receptors at clinically relevant concentrations (Belelli et al., 2003). The estimated effective plasma levels of etomidate correlate well with the concentrations required for modulation of $GABA_A$ receptors (Krasowski and Harrison, 1999).

The activity of etomidate is enantioselective and highly dependent on β_2 or β_3 subunit incorporation into $GABA_A$ receptors (Hill-Venning et al., 1997; Belelli et al., 2003). This selectivity is apparently conferred by an asparagine residue in position 265 (β_2N265) in the M2 domain, which distinguishes this ligand from pentobarbital (McGurk et al., 1998; Belelli

et al., 2003). The same residue is crucial for the activity of loreclezole (Wingrove et al., 1994; see also chapter 2.7.1.).

In vivo, N265 also determines the β-selectivity for etomidate (Belelli et al., 1997). Knock-in mice (β_3S265M) showed a reduced sensitivity to anaesthetic effects of etomidate and propofol (Jurd et al., 2003). It remained uncertain, whether these residues contribute to anaesthetic binding or allosteric modulation of gating. By means of a photoreactive analogue of etomidate ([^{3}H]azietomidate) amino acids contributing to a putative anaesthetic binding site were identified. The identified residues, α_1M236 and β_1M286, are likely to border a pocket in the transmembrane domain (located at the same subunit interfaces as the GABA binding site) (Li et al., 2006). The subunit stoichiometry of 2α:2β:1γ (Chang et al., 1996; Tretter et al., 1997) and the arrangement of $GABA_A$ receptor subunits (Baumann et al., 2002) predicts two interfacial etomidate sites per channel (Stewart et al. 2008).

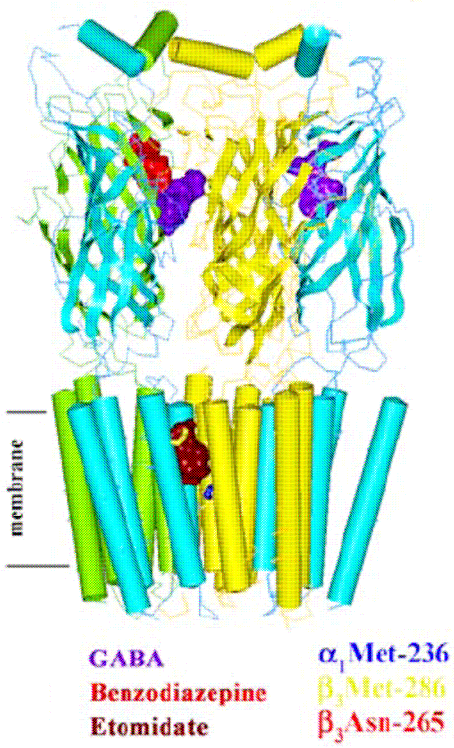


Figure 10 Illustration of an azietomidate binding site in a $GABA_A$ receptor homology model. Pockets in proximity of the binding sites for etomidate (maroon), GABA (purple), and benzodiazepines (red) are shown (Li et al., 2006).

Proposed orientation of etomidate in its binding pocket

The N3 atom of etomidate and the side-chain amine group of β_2Asn265 in the M2 region presumably form a hydrogen-bond. The imidazole ring of Trp237 in the M1 region and the phenyl group of etomidate were proposed to interact via a π–π stacking. Other residues located in the putative binding pocket include Ile230, Leu231, Thr233, Ile234 and Ser236 in

M1, Met261 in M2, and Phe293, Leu296, Leu297, and Ala300 in M3 (Campagna-Slater and Weaver, 2007).

2.3.2 PROPOFOL

The intravenous anaesthetic propofol increases the chloride conductance and the open probability of $GABA_A$ channels (Hales and Lambert, 1991). As reported for other anaesthetics e.g. methohexital (Koltchine et al., 1996), etomidate (Uchida et al., 1995; Sanna et al., 1997), alphaxalone (Horne et al., 1993), or isoflurane (Harrison et al., 1993; Mihic et al., 1994), propofol action does not require γ subunits (Jones et al., 1995). There is evidence for an involvement of the segment M3 in propofol binding (Bali and Akabas, 2003). β_2M286 has been suggested to play a major role, as mutating this residue abolished I_{GABA} enhancement without affecting direct activation (Krasowski et al., 1998). Mutation of β_2Tyr157, a residue located in the GABA binding site, decreases the direct receptor activation by propofol, but did not affect the potentiating activity (Fukami et al., 1999). These findings suggest that propofol acts via two distinct sites on $GABA_A$ receptors (Bali and Akabas, 2003). Residues potentially contributing to the binding site for propofol on $GABA_A$ receptors were identified by analysing the crystal structure of two propofol molecules bound to human serum albumin. The phenolic hydroxylgroup bonds to a backbone carbonyl at one site and a serine hydroxyl at the other side (Bali and Akabas, 2003).

Campagna-Slater and Weaver (2007) proposed that propofol on $GABA_A$ receptors is located in close proximity to the membrane-extracellular interface between M1 and M4, close to M3. The propofol hydroxyl-hydrogen atom might form a bond to the side-chain carbonyl group of β_2Gln224. Moreover, a π–π stacking interaction has been suggested to occur between the phenyl ring of residue Tyr448 and in addition a weak hydrogen-bond between the Tyr448 side-chain hydroxyl hydrogen atom and the propofol hydroxyl oxygen atom. Residues Gln224 and Tyr448 are conserved in all β subunit isoforms. This is consistent with the lack of β-subunit specificity of propofol. Other residues that have been suggested to interact with propofol molecule include Leu223, Thr225 and Tyr226 in M1, Leu285, Phe289 and Phe293 in M3, and Tyr444 and Trp445 in M4. The binding cavity for propofol has been suggested to open between the M1 and M4 segments on the extracellular side of the membrane, thus not the whole molecule is buried in the membrane, but one of the isopropyl groups might extend out in this opening (Campagna-Slater and Weaver, 2007). Mutation of residue β_2Y444W selectively suppresses the ability of propofol to enhance $GABA_A$ receptor function while normal sensitivity to etomidate and pregnenolone is retained. Propofol's reduced sensitivity

in this mutant is in line with the loss of a hydrogen bond within the propofol binding site (Richardson et al., 2007).

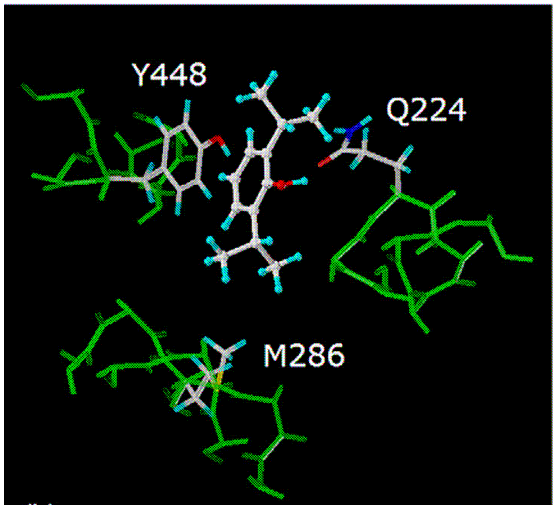


Figure 11 The docked propofol molecule in the putative binding site on the β_2 subunit on $GABA_A$ (Campagna-Slater and Weaver, 2007).

In vivo, β_3M265S knock-in mice propofol failed to show any anaesthetic effect (Jurd et al., 2003), although the action of propofol is independent of a particular β-subunit variant (Hill-Venning et al., 1997).

2.3.3 VOLATILE ANAESTHETICS

The CNS depressant effects of volatile anaesthetics have first been assumed to be non-specific actions (Janoff et al., 1981). At clinically relevant concentrations $GABA_A$ and glycine receptor mediated currents are enhanced (Franks and Lieb, 1994). Interestingly, the effect on $GABA_C$ receptors has been shown to be less pronounced (Mihic et al., 1997).

The size of the putative binding site for volatile anesthetics has been proposed to range between 250 and 370Å. Amino acids αL232, αS270, and αA291 (M1, M2, and M3, respectively) define a binding cavity for small anesthetic molecules (Jenkins et al., 2001).

Jenkins et al. (2001) reported later that the data for mutants L232F and S270H suggest that the anesthetics do not fit into the putative binding cavity in an identical manner. Mutation L232F did not affect isoflurane, while the enhancement of halothane was reduced. Halothane (unlike isoflurane) was active at the S270H mutant, suggesting a closer contact with L232 than isoflurane, which may instead be in close contact to S270. Introducing bulkier residues in

these positions results in a loss of sensitivity to both isoflurane and halothane (Jenkins et al., 2001).

αSer270 is likely to be involved in binding ethanol and isoflurane, as the $GABA_A$ receptors carrying the α_2S270C mutation were irreversibly enhanced by an alkanethiol anesthetic and the sulfhydryl reagent propyl methanethiosulfonate. The alkylated the α_2S270C mutant was insensitive to isoflurane (Mascia et al., 2000) suggesting that α_2S270 is indeed involved in the binding of volatile anaesthetics such as isoflurane (Jenkins et al., 2001). Isoflurane and sevoflurane evoke currents in the absence of GABA and similar to picrotoxin induce an open-channel block (Hasenender et al., 2002). Isoflurane and picrotoxin even might share a common binding site in the channel pore (Edwards and Lees, 1997), as picrotoxin suppresses the induction of a tail current on the removal of isoflurane, whereas bicuculline lacks this effect (Haseneder et al., 2002).

2.4 NEUROSTEROIDS

In the 1940s, metabolites of the hormone progesterone were identified as potent sedatives and anaesthetics (Seyle, 1941; Seyle, 1942). As the central depressant effect of such steroids occurs with a rapid time course, a genomic mechanism of action was excluded (Lambert et al., 2003).

Steroids have later been established as highly selective and potent positive allosteric modulators of $GABA_A$ receptors in multiple *in-vitro* experiments (Lambert et al., 2003).

Although endocrine glands such as ovaries and adrenals represent relevant sources of endogenous $GABA_A$ receptor active steroids, there is, however, evidence that certain neurons and glial cells possess the enzyme repertoire required for local synthesis of steroids. The brain may thus function in a paracrine fashion by locally influencing inhibitory neurotransmission. The mechanism by which neurosteroids are released *in-vivo* from neurons and glia is yet not fully understood (Lambert et al., 2003). Steroids do not affect $GABA_A$ channel single conductance, but they greatly facilitate the open state of $GABA_A$ receptors (Lambert et al., 1995).

Endogenous neurosteroids are potent modulators of all $GABA_A$ receptor isoforms. $GABA_A$ receptors are sensitive to low nanomolar concentrations of neurosteroids that have been shown to occur physiologically. At such concentrations (observed during stress, alcohol intoxication and oestrus) they potentiate GABA-induced currents, while at submicromolar to micromolar concentrations, which occur e.g during parturition, they gate the channel directly

(Lambert et al., 2003; Hosie et al., 2006). Thus, the major inhibitory neurotransmitter receptor in the mammalian CNS might be affected by both remote endocrine and local paracrine "fine-tuning" by neuroactive steroids (Lambert et al., 2003). *In-vivo* neuroactive steroids exhibit a behavioural profile resembling that of other positive allosteric modulators of $GABA_A$ channels. Neurosteroids are anxiolytic, anticonvulsant and sedative, and at higher concentrations neuroactive steroids induce general anaesthesia (Rupprecht, 2003).

To regulate $GABA_A$ receptor function neurosteroids require a C3α hydroxylgroup on the A-ring and a C20 ketone in the D-ring, indicating possible hydrogen bonding with polar and/or charged residues (Harrison et al., 1987).

2.4.1 INFLUENCE OF THE SUBUNIT COMPOSITION ON NEUROSTEROID ACTION ON $GABA_A$ RECEPTORS

In contrast to benzodiazepines (Sieghart, 1994; Sieghart, 1995) the modulatory effect by neurosteroids does not depend on a particular α- isoform (α_{1-6}) (Belelli et al., 2002). 3α,5β-5β-pregnan-3α-ol-20-one (3α, 5β- THPROG) produces a 6-7 fold enhancement of I_{GABA}. However, on receptors containing α_6 subunits the enhancement was markedly stronger (12-fold), suggesting a preference of neuroactive steroids for receptors containing α_6 subunits. The apparent affinity for neuroactive steroids is not affected by the α-isoform present (Belelli et al., 2002).

Small differences in apparent affinities might be of relevance, however, considering the concentrations of neurosteroids known to occur physiologically (Lambert et al., 2003). The relative insensitivity of $\alpha_4\beta_1\gamma_2$ receptors is of particular interest, since following progesterone withdrawal expression of this receptor subtype is increased. This up-regulation is apparently not mediated by progesterone itself, but by the $GABA_A$ receptor active metabolite 3α, 5β-THPROG (Follesa et al., 2001). The steroid sensitivity of $GABA_A$ channels containing α_4 subunits is strongly dependent on the subunits co-expressed, as $\alpha_4\beta\delta$ containing receptors are highly sensitive for neurosteroids (Belelli et al., 2002).

The β-variant has only little influence on the modulatory effect of neurosteroids (Sanna et al., 1997 Belelli et al., 2002, Lambert et al., 2003) and expression of a γ-subunit is not a prequisite for neurosteroid modulation of $GABA_A$ receptors. Replacement of γ-subunits by δ-subunits has been demonstrated to increase the sensitivity of $GABA_A$ receptors for steroids (Belelli et al., 2002). Receptors composed of α_4, β_3 and δ (a subunit combination expressed in the thalamus) are highly sensitive to steroid modulation compared to the corresponding γ2-

containing receptor (Belelli et al., 2002). In δ(-/-) mice neurosteroids fail to show anticonvulsant and anaesthetic properties (Mihalek et al., 1999; Lambert et al., 2003). There is evidence that receptors containing ε-subunits are spontaneously active. $GABA_A$ receptors containing ε-subunits were directly activated by steroids. These results are, however, difficult to interpret, because it is not clear whether the observed effect is due to direct gating of $GABA_A$ channels or a modulation of the spontaneous channel activity (Belelli et al., 2002; Thompson et al., 2002; Lambert et al., 2003).

2.4.2 THE NEUROSTEROID BINDING SITE

An azide derivative of 3α, 5β- THPROG did not directly label $GABA_A$ receptor subunits, but was incorporated into the voltage-dependent anion channel-1 (VDAC-1) (Darbandi-Tonkabon et al., 2003; Lambert et al., 2003).

Insights into the putative binding site of neurosteroids on $GABA_A$ receptors have been gained in a studied performed by Hosie et al. (2006), who replaced the murine M1 domain by the M1 domain of the Drosophila melanogaster RDL (resistance to dieldrin) $GABA_A$ receptor (RDL $GABA_A$ receptors exert a low sensitivity to potentiation and lacks direct activation by neurosteroids). Substitution of polar residues in the α_1 subunit by the homologous RDL positions identified αThr236 and αGln241 as crucial determinants of steroid action. Mutation αThr236Ile reduced receptor activation by α-tetrahydrodeoxycorticosterone (THDOC) and allopregnalone (ALLOP), whereas mutating αGln241 to Trp reduced the I_{GABA} potentiation and decreased the apparent agonist efficacy without affecting the half-maximal receptor activation. The effects of neurosteroids on $GABA_A$ channel function i.e. potentiation of GABA-gated currents and direct gating are therefore assumed to be mediated via 2 different binding sites.

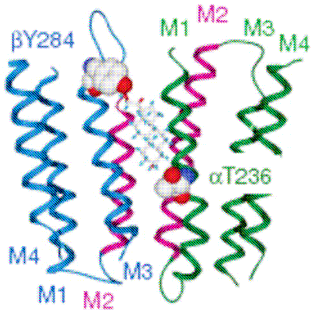


Figure 12 Model of the transmembrane domains of $GABA_A$ receptors with a bound THDOC molecule to αThr 236 and βTyr 284 (Hosie et al., 2006)

In analogy to a homology model of the transmembrane region of the nAch-receptor it was assumed that Thr236 lies on the receptor surface of the $GABA_A$ receptor close to the β/α

subunit interface (the same interface in which GABA binds). This residue would thus be accessible to hydrophobic molecules in the membrane. Gln241 lies at the base of a putative cavity between the α-subunits M1 and M4 domains. Upon receptor activation the depth and the volume of this cavity apparently increase and thus would allow the neurosteroid to bind to αGln241 and to stabilize the receptor in an active state. The relative locations of αThr236 and αGln241 on opposite faces of the M1 helix strongly suggest that they will contribute to steroid binding at 2 distinct sites. The neurosteroid's C20 ketone group can only participate as hydrogen-bond acceptor, whereas the C3α-hydroxylgroup can donate a hydrogen bond to Gln 241. The C20 ketone might also interact with the Asn407 and Tyr410 (M4 of the α1 subunit). Both of them can donate hydrogen bonds. Introduction of hydrophobic amino acids αAsn407Ala and αTyr410Phe reduces neurosteroid potency (Hosie et al., 2006). However, the mutation αAsn407Ala caused a greater reduction in the potency of THDOC (approx. 10-fold) than in that of ALLOP (approx. 3-fold), while αTyr410Phe solely reduced the potency of THDOC, which is consistent with the coordination of the oxygen moieties on the neurosteroid's D ring, the only region where THDOC and ALLOP structurally differ.

As mutation αGln241Trp did not only abolish the potentiating effect, but also disrupted the neurosteroid-induced direct activation, it was assumed that binding to αThr236-βTyr284 only elicits low-efficiency activation. High-efficiency activation depends on further binding to αGln241 and αAsn407, thereby potentiating the neurosteroid-induced activation (Hosie et al., 2006).

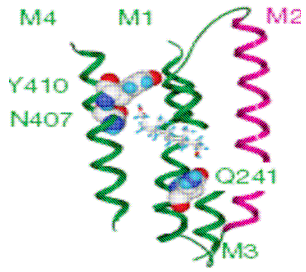


Figure 13 Illustration of the α subunit's transmembrane domains, showing α Gln 241, α Asn 407 and α Tyr 410 docking with a THDOC molecule (Hosie et al., 2006).

It is thus assumed that 2 discrete groups of residues in the $GABA_A$ receptor transmembrane domains mediate the effects of neurosteroids: binding to αThr236 and βTyr284 initiates the activation, while via αGln241 and αAsn407 the potentiation of responses to GABA and/or neurosteroids are mediated. Given the stoichiometry of $GABA_A$ receptors, two copies of these binding sites are apparently present. The above-mentioned residues are conserved throughout

the α and β subunits, thus they are ideally placed to mediate neurosteroid modulation and activation of all $GABA_A$ receptor subtypes (Hosie et al., 2006).

2.5 ETHANOL

The effects of ethanol on humans are well known. At low blood concentrations ethanol induces a feeling of euphoria and disinhibition, with increasing levels of ethanol motor function becomes impaired and speech becomes slurred. At blood ethanol concentrations reaching between 200 and 300 mg/dl blood, vomiting and falling into stupor can occur. Blood concentrations exceeding 300 mg/dl can induce coma, and at even higher concentrations (500mg/dl), there is a potential danger of respiratory failure and death (Davies, 2003).

Until it was shown that ethanol binds to specific proteins, in particular to ligand-gated ion channels and voltage-gated Ca^{2+}-channels, the lipid theory has been widely accepted (Tabakoff and Hoffmann, 1996; Davies, 2003).

In native receptors from rat brain microsacs and synpatoneurosoms enhancement of I_{GABA} by ethanol was shown for the first time. Ethanol exposure augmented the GABA-gated chloride uptake by 260% (Suzdak and Paul, 1987). Later, Wafford and Whiting (1992) showed that potentiation of I_{GABA} by ethanol was critically dependent on the presence of the γ_{2L} splice variant in $\alpha_1\beta_2\gamma_2$, whereas others failed to reproduce this finding (Sigel et al., 1993, Zhai et al., 1998). $GABA_A$ receptors containing $\alpha_{4/6}$, β_3 and δ subunits, those known to mediate tonic currents, have been described to be exceptionally sensitive to low ethanol concentrations (Wallner et al., 2003; Hanchar et al., 2005, Santhakumar et al., 2007). $GABA_A$ receptor δ-subunit knockout (δ-/-) mice show multiple defects in behavioural respones to ethanol (Mihalek et al., 2001). The α_6R100Q mutation, identified in alcohol-tolerant rats, is sufficient to induce ethanol hypersensitivity, to confer supersensitivity to tonic currents in rat cerebellar granule cells and to dramatically increase the ethanol sensitivity of recombinantly expressed receptors (α_6R100Q$\beta_3\delta$) (Hanchar et al., 2005). Therefore, an amino acid residue important for BZ sensitivity is also critical for low-dose ethanol sensitivity of α_6 containing $GABA_A$ receptors (Wallner et al., 2006). The imidazobenzodiazepine Ro15-4516, a structural analogue of the clinically used benzodiazepine antagonist flumazenil, is also an ethanol antagonist (Bonetti et al., 1988).

The question whether Ro15-4513 ‘s alcohol antagonism is related to a specific alcohol counteracting action or due to a non-specific functional antagonism or due to the weak inverse agonistic activity of Ro15-4513 on certain $GABA_A$ receptor subtypes has been overcome by identification of a high affinity binding site on $\alpha_4\beta_3\delta$ receptors. At anaesthetic and potentially

lethal concentrations (≥ 100mM), however, an additional Ro15-4513-insensitive component of ethanol enhancement occurs. This component is abolished by the mutation β_3N265M (Wallner et al., 2006). Ethanol increases the frequency and the duration of single-channel openings, and further augments channel bursts and burst duration and reduces the fraction of time that the channel remains in the closed state (Tatebayashi et al., 1998).

Various alcohols including methanol, butanol, hexanol, octanol, decanol and dodecanol enhance GABA-gated currents. A correlation between chain length and potency has been suggested, as ethanol potency increases with the number of carbon atoms in the backbone. However, alcohols possessing more than 12 carbon atoms do not affect GABA-gated currents anymore (Davies, 2003), indicating that they share a common binding site with a defined volume that accommodates only alcohol molecules with chain lengths not exceeding 12 carbon atoms (Nakahiro et al., 1996).

2.6 CATIONS MODULATING $GABA_A$ RECEPTOR FUNCTION

There is evidence for specific binding sites for divalent cations on ligand-gated ion channels. Calcium and other divalent cations have been shown to enhance or inhibit [^{3}H] GABA or [^{3}H] benzodiazepine binding (Lo and Snyder, 1983; Schwartz et al., 1994), though a direct interaction of calcium with $GABA_A$ channels has not be shown (Schwartz et al., 1994).

Magnesium attenuates neuronal excitability by a blockade of NMDA receptors under resting conditions (Mayer et al., 1984). Magnesium, thus, exhibits neuroprotective actions following seizure activity and cerebral ischemia (Tsuda et al., 1991; Schwartz et al., 1994). Magnesium also reduces basal and GABA-inhibited [^{35}S] TBPS binding to $GABA_A$ receptors in a dose-dependent manner up to a concentration of 1mM. Concentrations exceeding 10 mM enhanced the binding. The effect of magnesium does not depend on the presence of a γ-subunit (Moykkynen et al., 2001).

Another cation of special interest in neuronal activity is zinc, being present in the cerebral cortex and the hippocampus, both enhancing and inhibiting neuronal excitability (Westbrook and Mayer, 1987; Xie and Smart, 1991). Zinc has been demonstrated to interact with a binding site located at the extracellular domain of NMDA receptors, thus inhibiting NMDA function (Westbrook and Mayer, 1987).

The blocking effect of Zn^{2+} ions on $GABA_A$ receptor has been intensively studied revealing a dependence on the species and maturity of the neurons as well as on the $GABA_A$ receptor subunit composition (Draguhn et al., 1990; Smart et al., 1991). $GABA_A$ receptors comprising α_6 and β_3 subunits have been shown to be most sensitive to zinc (Burgard et al., 1996;

Knoflach et al., 1996; Saxena and MacDonald, 1996). Replacement of the δ subunit by a γ subunit as well as replacement of α_6 by α_1 greatly reduces inhibition of $GABA_A$ receptors by Zn^{2+} (Draguhn et al., 1990; Saxena and MacDonald, 1996; Korpi et al., 2002). Comparison of zinc's inhibitory effects on $GABA_A$ receptors comprising different α subunits revealed a higher sensitivity but lower efficacy of zinc on $GABA_A$ receptors containing α_1 subunits than on those containing α_2 or α_3 subunits (White and Gurley, 1995). It is assumed that the zinc binding site is formed by different subunits (Korpi et al., 2002). Wooltorton et al. (1997) reported that Zn^{2+} inhibition is significantly reduced by the mutation H292A in the M2 region in both homomeric β_3 and β_1 receptors indicating an involvement of this residue, that is located in the putative channel-lining M2 region, in determining zinc sensitivity (Korpi et al., 2002). Another amino acid residue located at the extracellular end of the M2 region (β_3H267) also contributes to zinc modulation of $GABA_A$ receptors (Hosie et al., 2003).

A major determinant of Zn^{2+} insensitivity for $GABA_A$ receptors containing γ subunits (N156) was found in the N-terminal extracellular domain of γ_{2L} subunits. This residue is highy conserved in all $GABA_A$ receptor subunit families except π. Interestingly, this amino acid is adjacent to H101 in the α_1 subunit, which is required for benzodiazepine sensitivity in ternary αβγ receptors (Nagaya and MacDonald, 2001). Other amino acids contributing to zinc insensitivity include residues γ_{2L}S278, γ_{2L}T279 and γ_{2L}I280 in the outer mouth of the M2 region as well as residue γ_{2L}K285 in the extracellular M2-M3 linker region (Korpi et al., 2002).

Lanthanum enhances GABA-gated currents neither via interaction with the benzodiazepine, barbiturate nor picrotoxin binding site, as no competition with chlordiazepoxid, pentobarbital or picrotoxin was observed (Ma and Narahashi, 1993). Lanthanum enhanced I_{GABA} through $\alpha_1\beta_2\gamma_2$ receptors, whereas a drastically reduced efficiency was observed for $\alpha_1\beta_2$ receptors (Im et al., 1992).

Copper inhibits GABA-induced currents in a non-competetive manner. The effect of copper cannot be influenced by cations such as lanthanum. There is evidence that zinc antagonizes the action of copper dose-dependently, suggesting that they share a common binding site. This binding site has been suggested to be located at or close to the external mouth of the channel (Ma and Narahashi, 1993).

2.7 OTHER MODULATORS OF $GABA_A$ RECEPTORS

2.7.1 LORECLEZOLE

Loreclezole is broad-spectrum anticonvulsant agent that has been shown to be effective against generalized absence seizures and tonic-clonic convulsions (Ghiani et al., 1996). Loreclezole enhances the effect of GABA by interacting with a specific modulatory site on the β-subunit. A 300-fold higher affinity for $GABA_A$ receptors containing β_2 or β_3 subunits over those comprising β_1 subunits was established (Wingrove et al., 1994). A single amino acid residue in the β_2 subunit (β_2N289 and β_3N290) was identified as crucial determinant for conferring loreclezole sensitivity. Mutation of this residue to the corresponding residue in the β_1 subunit (β_1S290) abolishes the effect of loreclezole (Wingrove et al., 1994).

2.7.2 MEFENAMIC ACID

The non-steroidal antiphlogistic mefenamic acid exhibits anticonvulsant and proconvulsant effects. There is evidence that this drug enhances I_{GABA} through $\alpha_1\beta_2\gamma_2$ receptors. However, it is inactive on the corresponding β_1 subunit containing subtype. Mefenamic acid gates $\alpha_1\beta_{2/3}$ directly, but blocks $\alpha_1\beta_1$ receptors. The positive modulatory action of mefenamic acid is determined by residue N290. When this position was mutated to serine (β_2N290S) the positively modulating effect is abolished. The reverse mutation in the β_1 subunit (β_1S290N) induces sensitivity for mefenamic acid. Thus, positive allosteric modulation, direct activation and inhibition are determined solely by the β-subunit variant present, as shown for homomeric β_1 and β_3 receptors (Halliwell et al., 1999).

2.7.3 CLOZAPINE

Clozapine is an atypical neuroleptic that inhibits $GABA_A$ receptors in brain vesicles with particular high efficiency in cerebrocortical and hippocampal areas. Clozapine has been demonstrated to block [^{3}H]muscimol and [^{3}H]SR 95531 binding. Receptors composed of α_1, β_2 and γ_2 subunits are sensitive to the antagonism by clozapine, whereas recombinant $\alpha_6\beta_{2/3}\gamma_2$ receptors are not inhibited. The $\alpha_6\beta_1\gamma_2$ subtype is however sensitive for clozapine, indicating that the effect of clozapine dependent on the β- subunit (in the order $\beta_1>\beta_2>\beta_3$). The effect of clozapine is not mediated via the benzodiazepine binding site, as flumazenil failed to block the effect of clozapine. It is assumed that clozapine blocks most $GABA_A$ receptors. The $GABA_A$ antagonism of clozapine is not shared by other neuroleptics such as haloperidol. The

effect of clozapine on $GABA_A$ receptors might account for the enhanced seizure susceptibility associated with clozapine treatment (Korpi et al., 1995).

2.7.4 FUROSEMIDE

Furosemide blocks $GABA_A$ receptors directly, an effect that is strongly dependent on the subunit composition (α_6 and $\beta_{2/3}$) (Korpi and Luddens, 1997). Furosemide antagonism does not depend on the presence of a γ or δ-subunit. Furosemide antagonism is not detectable in hippocampal and thalamic areas, although a modulation of $\alpha_4\beta_2\gamma_2$ receptors has been shown (Knoflach et al., 1996; Korpi and Luddens, 1997).

The mechanism of action, however, differs apparently from the mechanism of action of other compounds antagonizing the function of $GABA_A$ receptors. The effect of furosemide on $GABA_A$ receptors can be blocked by high concentrations of Ro5-4864 (Korpi and Lüddens, 1997). In ANT rats (carrying point mutation α_6R100Q; see also chapter 2.5.) the effect of furosemide was less pronounced. This point mutation does not account for the reduced activity, as furosemide antagonism was comparable on recombinant wild type and mutated $GABA_A$ receptors (α_6R100Q) (Makela et al., 1996). Based on the observation that furosemide preferentially modulates $GABA_A$ receptors containing α_6 subunits, molecular determinants for the activity of furosemide were identified: Mutation of I288T in the α_6 subunit largely shifts the IC_{50} values (10-fold reduction). This residue is apparently located in the M1 domain, thus suggesting that M1 with the isoleucine residue might contribute to a putative binding site for furosemide (Thompson et al., 1999). The effect of furosemide is strongly dependent on the β-subunit incorporated into the receptor and stronger effects on $\beta_{2/3}$ containing receptors are observed compared to β_1 containing channels (Korpi et al., 1995). Co-expression of β_1S265M and β_3N265S with α_6 and γ_2 subunits, respectively, showed that the effect of furosemide depends on the same residue as the action of loreclezole (see also chapter 2.7.1.).

Residue αI288 is presumably located in M1 and residue β_3N265 in the extracellular end of the M2 domain. These residues are likely to be located in close proximity at the extracellular end of the channel. In the closed state of the channel M1 segments might intercalate between M2 segments at the extracellular end of the receptor. Upon activation of the receptors, the movements of the M1 and the M2 region might flip a gate (as proposed for the muscle nicotinic receptor (Akabas and Karlin, 1995)). It is assumed that furosemide by direct interaction with the M1 segment stabilizes the closed state of the gate of $GABA_A$ receptors (Thompson et al., 1999).

3. TONIC AND PHASIC INHIBITION

Whereas phasic inhibitory synaptic neurotransmission is well characterized, there is also convincing evidence for tonic, non-synaptic communication (Macdonald et al., 2004).

Low concentrations of ambient GABA apparently activate extrasynaptic receptors causing a small, but persistent chloride current, which results in tonic inhibition enabling the neuronal excitability to be regulated (Mody, 2001). α_6-subunit containing receptors are found exclusively in cerebellar granule cells and the dorsal cochlear nucleus (Fritschy and Brunig. 2003; Sieghart and Sperk, 2002). They can therefore not account for the high GABA sensitivity associated with tonic inhibition in hippocampal pyramidal cells. The observed high GABA potency indicates the presence of δ-subunit containing receptors (Mortensen and Smart, 2006), as recombinant $\alpha_4\beta_3\delta$ receptors are highly sensitive to GABA (Brown et al., 2002).

αβ receptors have been shown to be at least 5-fold more sensitive to GABA than αβγ receptors (Baburin et al., 2008), raising the possibility that αβ receptors may also contribute to tonic inhibition.

Mortensen and Smart (2006) have shown that $GABA_A$ receptors lacking δ or γ subunit are likely to be expressed in low numbers in the extrasynaptic membranes of pyramidal neurons.

The extent to which extrasynaptic $GABA_A$ receptors are tonically active is affected by the $GABA_A$ receptor subtypes expressed and their affinity to GABA, the basal GABA concentration around the extrasynaptic domains e.g. due to a spillover from nearby GABAergic synapses and finally, the activity of GABA transporters that will regulate ambient GABA concentrations (Mortensen and Smart, 2006). Estimates of ambient GABA concentrations range from tens of nanomolar to a few micromolar (Lerma et al., 1986; Tossman et al., 1986). Endogenous GABA concentrations have apparently a highly non-uniform distribution between the synaptic and extrasynaptic areas (Mortensen and Smart, 2006). A variation in subunit composition of extrasynaptic receptors may include the α_5 subunit, which is expressed in high density in the hippocampus (Pirker et al., 2000) and shows a diffuse extrasynaptic distribution (Crestani et al., 2002). This would agree with recent evidence supporting an important role of $\alpha_5\beta\delta$ receptors in tonic inhibition in hippocampal pyramidal cells. The number of αβ receptors present in extrasynaptic receptors is rather low (estimated up to 10%) (Mortensen and Smart, 2006).

4. PATHOPHYSIOLOGICAL CONDITIONS ASSOCIATED WITH IMPAIRED GABAERGIC NEUROTRANSMISSION

GABA plays a major role in controlling the excitability of the brain. A disruption of the balance between excitatory and inhibitory neurotransmission is associated with multiple neurological disorders (Sieghart and Sperk, 2002).

An enhancement of the GABAergic system is associated with anxiolysis, sedation, amnesia, ataxia and loss of consciousness, whereas a reduction of the inhibitory GABAergic neurotransmission results in arousal, anxiety, restlessness, insomnia, exaggerated reactivity, and even epileptic seizures (Mohler, 2006). Impaired $GABA_A$ receptor function is associated with neurological disorders such as insomnia, anxiety disorders, epilepsy and schizophrenia (Sieghart and Sperk, 2002; Mohler, 2006).

4.1 INSOMNIA

Insomnia represents the most common sleep disorder affecting almost 20-50% of older adults (McCrae et al., 2007). Individuals suffering from sleep disorders can be grouped into three categories: sleep onset insomnia, sleep maintenance insomnia and terminal insomnia (awaking early morning combined with inability to return to sleep) (Rosenberg, 2006). GABAergic neurotransmission contributes significantly to human sleep regulation (Agosto et al., 2008). Three different areas of the central nervous system are involved in regulating non rapid-eye-movement sleep. Sleep-promoting GABAergic neurons in the ventrolateral preoptic nucleus (VPLO) are assumed to be tonically inhibited via noradrenergic neurons located in the locus coeruleus of the pons, thus inhibition of neurons of the locus coeruleus results in activation of VLPO neurons and thus may induce sleep. Moreover, the subsequent release of GABA from VPLO neurons is assumed to inhibit wake-active histaminergic neurons in the tuberomammilary nucleus (TMN) of the hypothalamus. The inactivation of the TMN seems to play a crucial role for the sleep onset (Bateson, 2004). Moreover, in a patient suffering from chronic insomnia a mutation in the gene encoding for the β_3 subunit was identified, the resulting subunit carrying in position 129 instead of an arginine a histidine residue (β_3R129H). Analysis of the $\alpha_1\beta_3$R129Hγ_2 receptors showed a slower rate of the phase of desensitisation and a faster current deactivation compared to wild type $\alpha_1\beta_3\gamma_2$ receptors (Buhr et al., 2002), giving further evidence for an involvement of decreased GABAergic neurotransmission in insomnia (Mohler, 2006).

4.2 ANXIETY

The precise pathogenesis underlying anxiety disorders is still not fully understood. Several distinct mechanisms apparently underlie different anxiety disorders, however, the GABAergic system is likely to be involved in anxiety. The GABAergic system has been suggested to play a major role in anxiety mainly based on the anxiety- relieving effects of benzodiazepines (Nutt and Malizia, 2001). γ_2-knockout mice exhibit dysfunctions of the GABAergic system including reduced benzodiazepine binding and receptor clustering in the cerebral cortex and the hippocampus, regions that are associated with anxiogenesis in humans (Crestani et al., 1999). Moreover, these mice reveal an increased behavioural reactivity to aversive stimuli and bias for threat cues, a behaviour that could be reversed by acute application of benzodiazepine site ligands. Thus, impaired GABAergic neurotransmission leading to enhanced harm-avoidance behaviour and enhanced sensitivity to negative associations might predispose individuals to generalized anxiety disorder (GAD) and panic disorder (PD) (Crestani et al., 1999; Nemeroff, 2003).

In patients suffering from anxiety disorders a reduction in GABA levels and benzodiazepine binding was detected, while in individuals suffering from PD a 22% reduction of GABA levels in the total occipital cortex was observed (Goddard et al., 2001). A global decrease of $GABA_A$ receptor binding was found throughout the CNS in untreated individuals suffering from PD compared to controls, in particular with most pronounced decrease in the orbitofrontal cortex and right insula, two areas associated with mediation of anxiety (Malizia et al., 1998), moreover also in the left hippocampus and the precuneus benzodiazepine binding was reduced (Bremner et al., 2000a).

In individuals suffering from posttraumatic stress disorder (PTSD) a similar reduction in $GABA_A$ receptor binding in the prefrontal cortex was observed, however without significant changes of binding in the pons, striatum, thalamus, cerecellum or midbrain compared to controls (Bremner et al., 2000b).

Moreover, also individuals suffering form GAD showed a reduced $GABA_A$ receptor binding in the left temporal lobe (Tiihonen et al., 1997).

4.3 EPILEPSY

Epilepsy is a neurological disorder affecting more than 0.5% of mankind. Several genetic factors are assumed to play a pivotal role in the development of generalized epilepsies (IGEs) and in some partial epilepsies (Kaneko et al., 2002). Mutations associated with epilepsy have

been identified in multiple voltage- and ligand-gated ion channels including sodium, calcium and potassium channels and nAch and $GABA_A$ receptors (Scheffer and Berkovic, 2003). Impairment of GABAergic neurotransmission by genetic mutations and/or application of $GABA_A$ receptor antagonists cause epileptic seizures, whereas drugs enhancing GABAergic neurotransmission represent an important class in antiepileptic therapy (Sperk et al., 2004).

4.3.1 MUTATIONS IN $GABA_A$ RECEPTOR SUBUNITS ASSOCIATED WITH EPILEPSY

Mutation	Epilepsy	Functional Consequences	References
γ2K289M	GEFS+	Shorter mean open times compared to wildtype receptors; reduces protein surface expression	*Baulac et al., 2001; Bianchi et al., 2002*
γ_2R43Q	CAE, febrile seizures	Significant reduction of the peak current amplitude; reduced surface expression of the receptor protein resulting from a receptor retention in the ER	*Wallace et al., 2001; Kang and Macdonald, 2004; Macdonald et al., 2004*
γ_2Q351X	GEFS+	Introduces a premature stop codon at position Q351; formation of non-functional receptors	*Harkin et al., 2002; MacDonald et al., 2004*
γ_2 IVS6+2T→G	CAE, FC	Results in a truncated γ_2 subunit protein; causes non-functional channels	*Macdonald et al., 2004*
α_1A322D	JME	Causes reduced peak current amplitudes, altered current kinetics and reduced mean channel open times; affects α_1 subunit trafficking after translation; due to the misfolding of the protein the mutant subunit is degraded in the ER (ERAD)	*Cossette et al., 2002;* Gallagher et al., 2005
δE177A, δR220H	GEFS+, JME	Reduces current amplitudes and receptor surface expression	*Feng et al., 2006*

GEFS+ Generalized epilepsy with febrile seizures plus

ER Endoplasmatic reticulum

FC Febrile convulsions

CAE Childhood absence epilepsy

JME Juvenile myoclonic epilepsy

4.3.2 TEMPORAL LOBE EPILEPSY

In patients suffering from temporal lobe epilepsy and animal models profound changes in $GABA_A$ receptors function have been observed such as a substantial cell loss in CA1 accompanied by a dramatic loss of $GABA_A$ receptors (Sperk et al., 2004; Mohler, 2006). In these cells subtype-specific changes in the subcellular distribution with an increase of α_1, α_2, α_3 and γ_2 were detected pointing to a potential sprouting of GABAergic axons in the epileptic dentate gyrus. The latter might represent a compensatory mechanism to the loss of GABAergic neurons in the dentate gyrus (Mohler, 2006). Interictal activity in individuals suffering from temporal lobe epilepsy is apparently linked to depolarising GABAergic synaptic events at pyramidal cells (Cohen et al., 2002). The depolarising GABA-mediated response is more likely caused by a very negative resting potential of these cells rather than by changes in the chloride gradient (Stein and Nicoll, 2003). This finding is of major importance, as approximately 50% of all clinically used antiepileptics owe their efficacy either totally or at least partially to potentiation of GABA-mediated responses (Mohler, 2006).

4.3.3 ABSENCE EPILEPSY

Interconnected networks of inhibitory neurons regulate oscillations throughout the CNS. One of these netoworks, the thalamic reticular nucleus (TRN), participates in many thalamo-cortical oscillations such as spindles in sleep and spike-wave seizures typically observed in generalized absence epilepsy. Similar to the generation of sleep spindles, spike-wave seizures may result from events, in which TRN neurons inhibit thalamocortical (TC) relay neurons, thereby inducing rebound bursts mediated by T-type channels resulting in a reexcitation of the TRN. TRN has been shown to be the source of the hyperpolarizing input that recovers calcium currents mediated by T-type channels in TC neurons from inactivation, suggesting that the TRN contributes to absence seizures.

The antiabsence drug clonazepam is known to suppress rhythmic activity in thalamic and thalamocortical slices. In the TRN, clonazepam acts on α_3 containing $GABA_A$ receptors as

well as in TC neurons on α_1 containing receptors, an enhancement which is required to suppress oscillations in thalamic slices (Sohal et al., 2003; Mohler, 2006).

4.4 SCHIZOPHRENIA

A deficiency in GABAergic inhibitory neurotransmission is assumed to contribute particularly to the symptoms of schizophrenia (Lewis et al., 2005). Changes in cortical GABAergic systems have been reported in post-mortem brains of individuals suffering from schizophrenia including reduced uptake and release of GABA and a decreased activity of the glutamic acid decarboxylase. The axon terminals of GABAergic chandelier neurons were insufficient in the prefrontal cortex as shown by a 40% reduction of GAT-1 (Woo et al., 1998; Mohler, 2006).

Additionally, there is evidence for an enhanced α_2-containing receptor expression in the axon initial segment of pyramidal cells, pointing to a synapse specific deficit of GABA-mediated neurotransmission in schizophrenia (Volk et al., 2002). The ratios of γ_2 subunit splice variants are apparently affected in the prefrontal cortex of schizophrenics (Huntsman et al., 1998). In the primate brain dopamine (D4) receptors regulate GABAergic interneurons in the cerebral cortex, hippocampus, thalamic reticular nucleus and globus pallidus. Thus, the beneficial effects of clozapine in the treatment of schizophrenia may be partially caused by a D4-mediated modulation of $GABA_A$ receptors (Mrzljak et al., 1996; Mohler, 2006).

The GABAergic control in the dopaminergic system is mainly mediated via α_3- containing receptors (Fritschy and Mohler, 1995; Pirker et al., 2000). α_3-/- mice display a significant deficit in prepulse inhibition of the acoustic startle reflex, underlying the deficit in sensorimotor information processing (Yee et al., 2005). After administration of the dopamine antagonist haloperidol this deficit was normalised, suggesting that this effect is mainly caused by an increased activity of the dopaminergic system (Yee et al., 2005). Compounds selectively interacting with α_3-containing receptors might be effective agents in treating the sensorimotor deficits in various psychatric diseases (Mohler, 2006).

There is evidence, that the hippocampus plays also an important role in the modulation of prepulse inhibition. In a α_5H105R point mutated mouse prepulse was diminished accompanied by an increase in spontaneous motor activitiy (Hauser et al., 2005).

5. $GABA_B$ RECEPTORS

The metabotropic $GABA_B$ receptor was first identified based on the receptor's distinct pharmacological profile compared to ionotropic $GABA_A$ or $GABA_C$ receptors (Hill and Bowery, 1981).

Subsequently, it was shown that $GABA_B$ receptors are G-protein coupled receptors (GPCR) that inhibit the adenylyl cyclase via the $G_{\alpha i/o}$ subunits of the activated G-protein (Hill, 1985).

There is evidence that $GABA_B$ receptors are involved in regulation of transmitter release at several synapses (Bettler and Tiao, 2006). $GABA_B$ receptors also regulate Ca^{2+}- and K^+-channels at both pre- and postsynaptic sites via the $G_{\beta\gamma}$ subunits of the G-Protein (Bowery et al., 2002, Calver et al., 2002; Bettler et al., 2004). Presynaptic $GABA_B$ receptors have been found on both inhibitory and excitatory terminals where they act as auto- and heteroreceptors, respectively. Upon activation of presynaptic $GABA_B$ receptors neurotransmitter release is suppressed via inhibition of voltage-gated Ca^{2+}-channels (Mintz & Bean, 1993; Poncer et al., 1997). Postsynaptic $GABA_B$ receptors have been shown to induce slow inhibitory postsynaptic currents (sIPSC) by activating Kir3-type K^+-channels, which induce a hyperpolarization of the membrane, thus postpone excitatory currents (Luscher et al., 1997).

$GABA_B$ receptors modulate synaptic plasticity, heterosynaptic depression, population burst firing and inhibition of backpropagating action potentials (Bettler and Tiao, 2006), thus they represent promising drug targets (Bettler et al., 2004, Cryan and Kaupmann, 2005; Bowery, 2006). Several indications are available for the $GABA_B$ agonist baclofen. However, the use of baclofen in humans is limited due to the rapid development of tolerance and adverse effects after systemic administration. Thus, much effort is put in the development of novel compounds that might overcome the shortcomings of the so far available $GABA_B$ receptor agonists (Bettler and Tiao, 2006).

So far, 2 molecular subtypes of $GABA_B$ receptors are established (Bettler et al., 2004, Bettler and Tiao, 2006). There is evidence that elusively upon assembly of $GABA_B1$ and $GABA_B2$ subunits functional $GABA_B$ receptors are formed (Marshall et al., 1999, Mohler and Fritschy, 1999; Bettler et al., 2004). Interaction of $GABA_B1$ with $GABA_B2$ subunits is not only obligatory for cell surface expression but also for robust G-protein coupling (Margeta-Mitrovic et al., 2000, Calver et al., 2001, Galvez et al., 2001).

The molecular diversity in the $GABA_B$ system mainly arises from the $GABA_B1a$ and $GABA_B1b$ subunit isoforms (Kaupmann et al., 1997) and there is also evidence for two isoforms for $GABA_B2$ (Bettler and Tiao, 2006). Structurally, they differ in their N-terminal domain by a pair of sushi repeats that is present in $GABA_B1a$, but not in $GABA_B1b$ (Blein et

al., 2004, Bettler and Tiao, 2006). This structural peculiarity -Sushi repeats- is also known as complement control protein modules, or short consensus repeats that have been found in other GPCR as well, play a role in mediating protein interactions in a wide variety of adhesion proteins (Bettler and Tiao, 2006).

$GABA_B$ receptors were the first example of GPCR that inevitably needs to heterodimerize to function. This unique property has been employed to study the mechanism underlying subunit assembly and receptor surface trafficking. It is well established that a C-terminal arginine-based ER retention/retrieval signal, RSRR, retains unassembled $GABA_B1$ subunits in the ER and limits surface expression to correctly assembled heteromeric receptors (Couve et al., 1998, Margeta-Mitrovic et al., 2000; Pagano et al., 2001, Bettler and Tiao, 2006). Most likely, the RSRR signal in $GABA_B1$ is inactivated by interaction of the $GABA_B1$ with the $GABA_B2$ subunit, which then triggers trafficking (Bettler and Tiao, 2006).

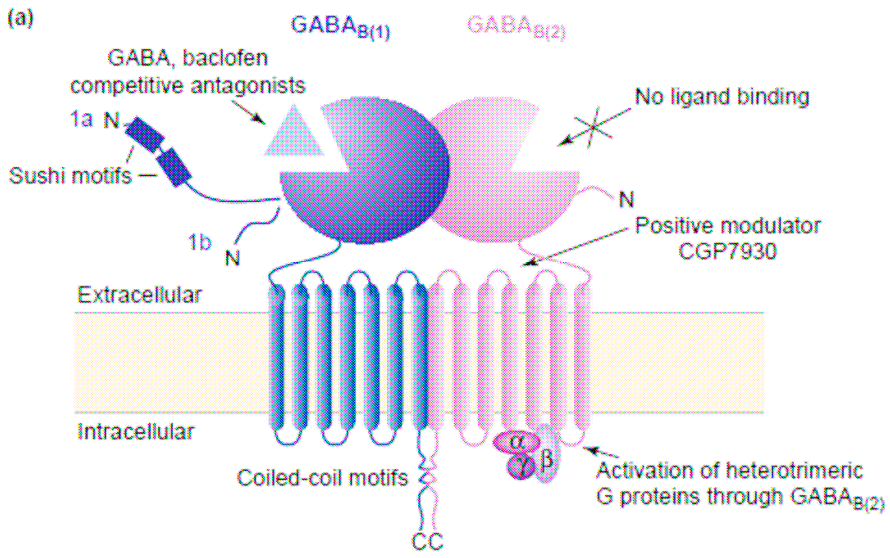


Figure 14 Schematic illustration of $GABA_B$ receptors as heterodimers of two subunits, $GABA_B1$ (dark blue) and $GABA_B2$ (violet) (Cryan and Kaupmann, 2005)

6. $GABA_C$ RECEPTORS, A $GABA_A$ RECEPTOR SUBTYPE

$GABA_C$ receptors gate Cl^--currents in various parts of the vertebrate brain. $GABA_C$ receptors were first described in interneurons of the spinal cord. Later, $GABA_C$ receptors were found in the retina (Enz and Cutting, 1998). Compared to $GABA_A$ receptors, $GABA_C$ receptors exhibit distinct electrophysiological properties: they are characterized by i.) higher sensitivity for GABA, ii.) smaller current amplitudes, iii.) they do not desensitise; iv.) prolonged single channel mean open times and v.) a lower chloride conductance (Feigenspan et al., 1993). $GABA_C$ receptors differ from $GABA_A$ and $GABA_B$ receptors in regard to their pharmacological properties. They are insensitive for typical $GABA_A$ receptor modulators such as benzodiazepines and barbiturates, and they are not blocked by bicuculline and they cannot be activated by the $GABA_B$ agonist baclofen (Bormann and Feigenspan, 1995).

It was, however, suggested to classify $GABA_C$ receptors as $GABA_A$ receptor subtype (Barnard et al., 1998; Olsen and Sieghart, 2008; Collingridge et al., 2008)

These receptors are composed of ρ subunits. A major characteristic of ρ subunits is their ability to build functional homooligomers (Enz and Cutting, 1998). Upon homooligomeric expression of ρ_2 subunits GABA-gated channels are formed, however, with markedly lower whole cell currents produced compared to ρ_1 receptors (10-100pA for ρ_2 versus 200-1000pA for ρ_1) (Wang et al., 1994). The ρ_3 as well as the white perch ρ subunit have been also demonstrated to form homooligomeric, functional GABA-gated channels with electrophysiological and pharmacological properties similar to that of native $GABA_C$ receptors (Enz and Cutting, 1998). Human ρ_1 subunits apparently do not assemble with $GABA_A$ receptor α_1, β_1, γ_2 and glycine receptor β subunits (Shimada et al., 1992). Besides electrophysiological and biochemical evidence, there is moreover anatomical data showing that $GABA_C$ receptor subunits do not interact with $GABA_A$ or glycine receptor subunits (Koulen et al., 1998).

$GABA_C$ receptors are sensitive to picrotoxinin (Enz and Cutting, 1998). In addition to the non-competitive inhibition of $GABA_C$ receptors by pore occlusion, picrotoxinin has been shown to display a competitive antagonism on $GABA_C$ receptors. Mutation P309S in the channel lining region enhances picrotoxinin sensitivity (Wang et al., 1995). A similar residue V257 in the $GABA_A$ receptor M2 domain is close to the picrotoxinin binding site or even directly involved in binding (Xu et al., 1995).

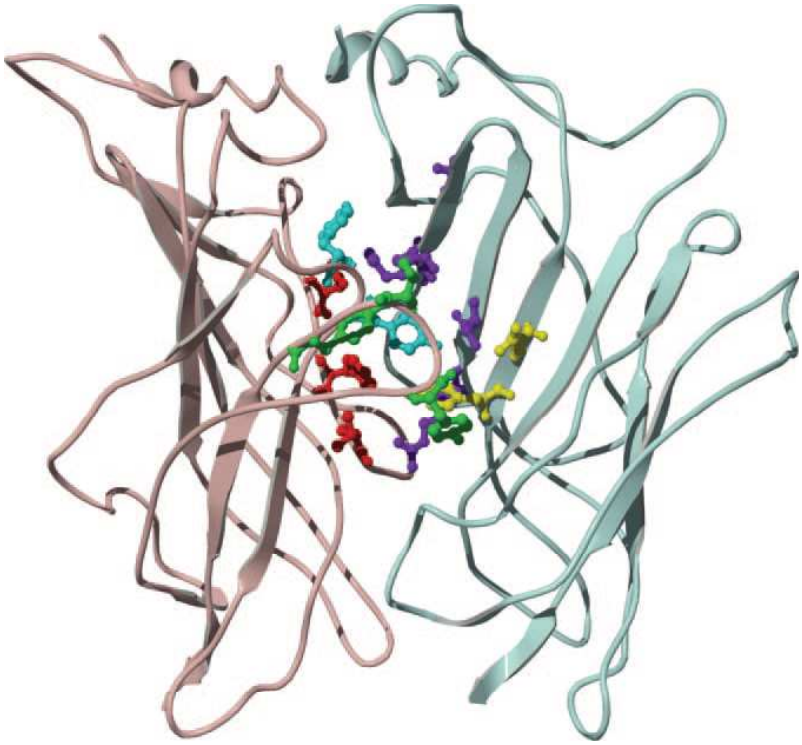


Figure 15 The agonist/antagonist binding site of $GABA_C$ receptors. In analogy to $GABA_A$ receptors the binding site is constituted by different loops (A-E) with amino acid contributing to the binding pocket. However, loop F in ρ_1 homomeric $GABA_C$ receptor is apparently not involved in agonist binding which stands in clear contrast to $GABA_A$ receptors (see also chapter 1.5.). Residues Asp136, Phe138, and Val140 in loop A (depicted in red), Tyr198 and Tyr200 in loop B (depicted in cyan), and Tyr241, Thr244, and Tyr247 in loop C (depicted in green) are assumed to form the principal site of the binding pocket, whereas residues Tyr102 and Arg104 in loop D (depicted in yellow) and Val155, Met156, Val159, Ser168, and Arg170 in loop E (depicted in purple) form the complimentary face of the binding pocket (Sedelnikova et al., 2006).

7. MODULATION OF $GABA_A$ RECEPTORS BY PLANT EXTRACTS AND NATURAL PRODUCTS

7.1 VALERIAN (VALERIANA OFFICINALIS)

Valerian root (*Valeriana officinalis* L.) is a traditionally used herbal drug with sedative and CNS depressant effects (Houghton, 1999). Its mechanism of action, however, is not known so far and its efficacy is discussed controversially (Bent et al., 2006; Trauner et al., 2008).

In order to get insight into the molecular mechanism of action and target of *Valerian* preparations and active constituents of *Valerian*, respectively, multiple investigations on different molecular targets including the GABAergic system (Santos et al., 1994; Cavadas et al., 1995; Ortiz et al., 1999; Yuan et al., 2004) or Adenosine type 1 (A_1) receptors (Schumacher et al., 2002) have been carried out (Trauner et al., 2008). Aqueous Valerian

extracts have been shown to enhance [^{3}H] GABA release due to a reversal of the GABA carrier (Santos et al., 1994). Cavadas et al. (1995) observed a displacement of [^{3}H] muscimol by aqueous and hydroalcoholic extracts of *Valerian*. Ethanolic *Valerian* extracts enhanced [^{3}H] flunitrazepam binding (Ortiz et al., 1999). Various flavonoids including 6-methylapigenin, 2S(-)-hesperidin and linarin displayed sleep-enhancing, sedative and anxiolytic properties in animal models (Marder et al., 2003; Fernandez et al., 2004). Previously, a *Valerian* extract and valerenic acid were shown to decrease the firing rate in brainstem neurons in a concentration dependent manner, an effect that could be antagonized by bicuculline (Yuan et al., 2004). In a previous study, valerenic acid was identified as a modulator of $GABA_A$ receptors containing β_2 or β_3 subunits (Khom et al., 2007; see chapters results and discussion). Benke et al. (2008) provided evidence for an *in-vivo* interaction of valerenic acid and valerenol with $GABA_A$ receptors. Both compounds displayed anxiolytic activity with high potencies in mice. In mice carrying the point mutation β_3N265M valerenic acid failed to show anxiolytic effects. It has thus been suggested that neurons expressing β_3 containing $GABA_A$ receptors represent a major cellular substrate for the anxiolytic action of valerenic acid (Benke et al., 2008).

7.2 BLACK COHOSH (ACTAEA RACEMOSA)

Preparations of *Black Cohosh* (*Actaea racemosa*) represent popular and frequently applied herbal products in the treatment of postmenopausal disorders; there is arising evidence from clinical trials that *Actaea racemosa* preparations containing actein affect CNS related symptoms of postmenopausal disorders including insomnia, restlessness and anxiety. The estrogenic effect of actein and *Black Cohosh* preparations remains controversially discussed (Cheema et al., 2007; McBane, 2008, Uebelhack et al., 2006).

There is accumulating evidence that *Black Cohosh* preparations are more effective than placebo in alleviating moderate vasomotor symptoms associated in the menopause (Cheema et al., 2007; McBane, 2008, Uebelhack et al., 2006). In particular, an improvement in sleep and mood disturbances was observed (Cheema et al., 2008).

Interactions with CNS targets of *Black Cohosh* and its main constituents have been suggested, including the central endogenous opioid system (Reame et al., 2008), the μ-opoid receptor (Rhyu et al., 2006), 5-HT_{1A}, 5-HT_{1D} and 5-HT_7 receptors (Burdette et al., 2003) or D_2 receptors (Jarry et al., 2003).

8. AIMS

Valerian and *Black Cohosh* are traditionally used as "phytopharmaceuticals" to treat insomnia and anxiety (*Valerian*) and postmenopausal disorders (*Black Cohosh*) including symptoms like restlessness and agitation. In order to analyse the basis of the suggested sedative and anxiolytic effects I studied the action of the extracts and some of their main constituents on $GABA_A$ receptors expressed in *Xenopus* oocytes under two-microelectrode voltage clamp.

Specific aims were:

1. To analyse the modulation of $GABA_A$ receptors by commercially available *Valerian* extracts.
2. To characterise the action of valerenic acid, acetoxy- and hydroxy-valerenic acid (natural compounds isolated from *Valerian*) on $GABA_A$ receptors.
3. To characterise the subunit-specific action of valerenic acid on $GABA_A$ receptors.
4. To study the modulation of $GABA_A$ receptors by *Black Cohosh* extracts.
5. To analyse the molecular mechanism of action of actein, a compound isolated from *Black Cohosh,* on $GABA_A$ receptors.
6. To investigate a possible subunit-specific action of actein
7. To study the *in vivo* action of actein in a mouse model (Open Field, Elevated Plus Maze, Light Dark Choice, Stress induced hyperthermia)

9. MATERIALS AND METHODS

9.1 CHEMICALS

Valerenic acid was from Extrasynthese, France, Lyon, acetoxy-valerenic acid from LGC Promochem, Germany, Wesel and hydroxy-valerenic acid was isolated from *Valerian* root (kindly provided by Univ. Prof. DDr. Brigitte Kopp). A first sample of actein was kindly provided by Univ. Prof. DDr. Brigitte Kopp. Actein was also obtained from LGC Promochem, Germany, Wesel and Herbstandard Inc., California, USA. The structural formula for valerenic acid, acetoxy- and hydroxy-valerenic acid and actein, respectively, are given in Fig. 18 and 26. *Valerian* Extracts C1, C2, C3, C4 and C5 and *Actaea racemosa* extracts A1, A2 and A3 were kindly provided by Univ.Prof. DDr. Brigitte Kopp and colleagues (Mag. Gabriele Trauner and Mag.Ulrike Jäger). Stock solutions (100 mM) were prepared in DMSO (Dimethyl Sulfoxide, Sigma, Austria). Because of low solubility in ND96, valerenic acid, the two derivatives and actein were only used up to a concentration of 300 µM. Equal amounts of DMSO were present in control and compound-containing solutions. The maximum DMSO concentration in the bath (0.3%) did not affect I_{GABA} (Khom et al., 2006; Khom et al., 2007). For experiments on mice, a stock solution of actein was prepared in 100% DMSO (50mg Actein/375 µl DMSO). Actein was further diluted in 0.9% sodiumchloride solution up to the final desired concentrations. Actein and control solutions were freshly prepared every day prior to experiments. Care was taken for equal amounts of DMSO in the actein and control solutions (Khom et al., in preperation).

9.2 ANIMALS

Male mice (C57Bl/6N) were obtained from Charles River Laboratories (Germany). For breeding and maintenance mice were group housed with free access to food and water.
Temperature was fixed to 23±1°C and 60% humidity with a 12 h light–dark cycle (lights on 0700–1900 hours). Male mice at 3–8 months age were tested in all experiments. All procedures involving animals were approved by the Austrian Animal Experimentation Ethics Board in compliance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes ETS no.: 123. Every effort was taken to minimize the number of animals used (Wittmann et al., 2008; Khom et al., in preparation).

9.3 EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF $GABA_A$ RECEPTORS

Preparation of stage V-VI oocytes from *Xenopus laevis*, synthesis of capped off run-off poly(A^+) cRNA transcripts from linearized cDNA templates (pCMV vector) was performed as described (Khom et al. 2006). Briefly, female *Xenopus laevis* (NASCO, USA) were anaesthetised by exposing them for 15 minutes to a 0.2 % solution of MS-222 (methane sulfonate salt of 3-aminobenzoic acid ethyl ester; Sandoz) before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/ml collagenase (Type 1A, Sigma). One day after isolation, the oocytes were injected with about 10-50 nl of DEPC- treated water (diethyl pyrocarbonate, Sigma, Germany) containing the different cRNAs at a concentration of approximately 150 - 3000 ng/µl/subunit. The amount of cRNA was determined by means of a NanoDrop ND-1000 (Kisker-biotech, Steinfurt, Germany).

Mutation β_{1S290N} was introduced by the "gene SOEing" technique (Horton et al., 1989). This involved synthesizing mutagenic oligonucleotides to introduce the desired mutation, and using a silent restriction site was used to screen for the mutation. The mutant cDNA was verified by sequencing.

To ensure expression of the gamma-subunit in the case of $\alpha_1\beta_2\gamma_1$, $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_1$ and $\alpha_6\beta_2\gamma_{2S}$ receptors, cRNAs were mixed in a ratio of 1:1:10 (Boileau et al., 2002b) except $\alpha_4\beta_2\gamma_{2S}$ (ratio 3:1:5). For receptors comprising only α and β subunits ($\alpha_1\beta_2$, $\alpha_2\beta_2$, $\alpha_1\beta_3$, $\alpha_1\beta_{2N265S}$ (cDNA gift of E. Sigel), $\alpha_2\beta_2$, $\alpha_3\beta_2$, $\alpha_5\beta_2$), the cRNAs were mixed in a ratio 1:1. cRNAs for of $\alpha_1\beta_1$ and $\alpha_1\beta_{1S290N}$ channels were injected in a ratio 3:1 to avoid formation of β_1 homooligomeric $GABA_A$ receptors (Krishek et al., 1996). Oocytes were stored at 18°C in ND96 solution (Methfessel et al., 1986). Electrophysiological experiments were done using the two-microelectrode voltage-clamp method at a holding potential of −70 mV making use of a TURBO TEC 01C amplifier (npi electronic, Tamm, Germany) and an Axon Digidata 1322A interface (Molecular Devices, Sunnyvale, CA). Using pCLAMP v.9.2 data acquisition was carried out. The bath solution contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$ and 5 mM HEPES (pH 7.4). Microelectrodes were filled with 2M KCl and had resistances between 1 and 3 MΩ (Khom et al., 2007).

9.4 PERFUSION SYSTEM

GABA and VA and GABA and actein, respectively were applied by means of fast perfusion system (see Baburin et al., 2006 for details; Khom et al., 2006). Drug or control solutions were applied by means of a TECAN Miniprep 60 permitting automation of the experiments. To elicit I_{GABA} the chamber was perfused with 120 µl of GABA-containing solution at volume rate between 300 and 1000 µl/s. The I_{GABA} rise time ranged between 100 and 250 ms (see Khom et al., 2006). Care was taken to account for possible slow recovery from increasing levels of desensitization in the presence of high GABA, valerenic acid and actein concentrations. The duration of washout periods was therefore extended from 1.5 minutes (1-20 µM GABA, <10 µM valerenic acid or actein) to 30 minutes (≥100 µM GABA, ≥10 µM valerenic acid and actein) respectively. Oocytes with maximal current amplitudes >3 µA were discarded to exclude voltage-clamp errors (Khom et al., 2006; Baburin et al., 2006; Khom et al., 2007).

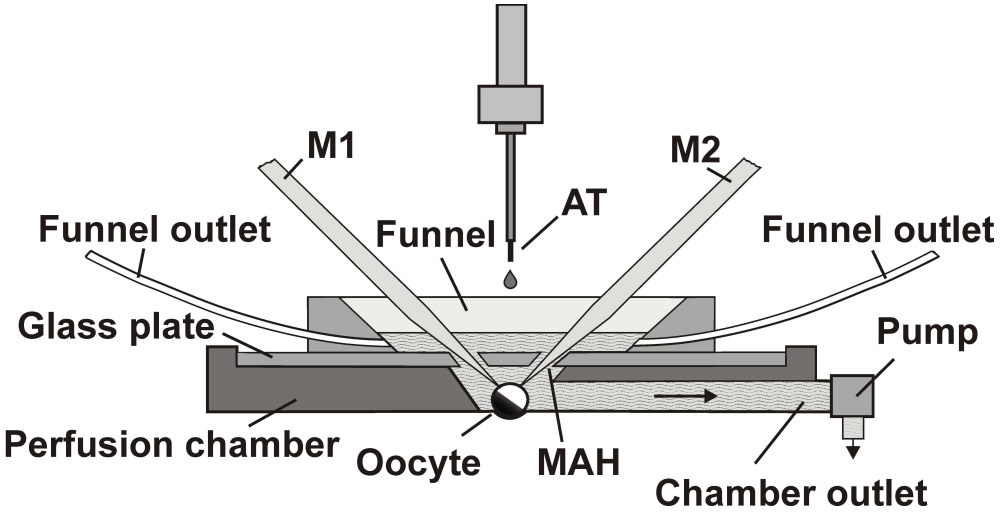


Figure 16 Cross-section view of the oocyte perfusion chamber. Two microelectrodes (M1 and M2) are inserted into the angular access inlets in a glass plate (the inlets serve simultaneously as perfusion holes) covering the small (15 µl) oocyte chamber. Drugs and control solutions were applied by a Tecan Miniprep 60 application tube (AT) to a quartz funnel surrounding the microelectrode access holes (MAH). Residual solution was removed from the funnel before drug application via the funnel outlets (Khom et al., 2006)

9.5 ANALYZING CONCENTRATION-RESPONSE CURVES

Stimulation of chloride currents by modulators of the $GABA_A$ receptor was measured at a GABA concentration eliciting between 5 and 10% of the maximal current amplitude ($EC_{5–10}$). The $EC_{5\text{-}10}$ was determined at the beginning of each experiment.

Enhancement of the chloride current was defined as $(I_{(GABA+Comp)}/I_{GABA}) - 1$, where $I_{(GABA+Comp)}$ is the current response in the presence of a given compound (valerenic acid or actein) and I_{GABA} is the control GABA current. To measure the sensitivity of the $GABA_A$ receptor for a given compound, it was applied for an equilibration period of 1 minute before applying GABA ($EC_{5\text{-}10}$). Concentration-response curves were generated and the data were fitted by non-linear regression analysis using Origin software (OriginLab Corporation, USA). Data were fitted to the equation: $\frac{1}{1+\left(\frac{EC_{50}}{[Comp]}\right)^{n_H}}$, where n_H is the Hill coefficient. Each data point represents the mean ± S.E. from at least 4 oocytes and ≥ 2 oocyte batches. Statistical significance was calculated using paired Student *t*-test with a confidence interval of $p < 0.05$ (Khom et al., 2007).

9.6 OPEN FIELD TEST

The open field test was performed as previously described by Wittmann et al. (2008). Briefly, the animals' behaviour was tested over 10 min in a 50x50 cm flexfield box equipped with infrared rearing detection. Illumination was set to 150 lux. Animals were video monitored and their explorative behavior was analyzed using the Video-Mot 2 equipment and software (TSE-systems, Bad Homburg, Germany). Arenas were subdivided into border (up to 8 cm from wall), center (20x20 cm, i.e. 16% of total area), and intermediate area according to the recommendations of EMPRESS (European Mouse Phenotyping Resource of Standardised Screens; http://empress.har.mrc.ac.uk) (Wittmann et al., 2008).

9.7 ELEVATED PLUS MAZE TEST

The elevated plus maze test was performed as previously described (Wittmann et al., 2008).

The animals's behavior was tested over 5 min on an elevated plus maze 1m above ground consisting of two closed and two open arms, each 50x5 cm in size. The test instrument was built from gray PVC; the height of closed arm walls was 20 cm. Illumination was set to 180

lux. Animals were placed in the center, facing an open arm. Analysis of open and closed arm entries and time on open arm was automatically done with Video-Mot 2 equipment and software (Wittmann et al., 2008).

9.8 LIGHT-DARK CHOICE TEST

The light-dark choice test was adopted and performed as previously described by Wittmann et al. (2008). Explorative behavior in a brightly lit area (400 lux) was investigated by insertion of a black box into the open field arena, covering one third of the space. Time spent and distance travelled was measured over a 10 min period in the open area. One small field directly at the entrance to the black box was assigned as transition zone. To reach the larger compartment assigned as open area, the mouse had to leave the dark area completely (Wittmann et al., 2008).

9.9 STRESS-INDUCED HYPERTHERMIA TEST

The stress-induced hyperthermia (SIH) test was performed as previously described (Wittmann et al., 2008). A temperature probe, lubricated with glycerol, was inserted into the rectum of the mouse for a depth of up to 2 cm. The temperature probe remained in the animal till a stable temperature was reached (maximum 10 s). Temperature measurement was repeated after 10 min and the rise in temperature between the first and second measurement was considered as stress-induced hyperthermia (Olivier et al., 2003).

9.10 HOME CAGE ACTIVITY

Basal activity and circadian rhythm of control and actein-treated mice was monitored in their home cages using the Infra-Mot system (TSE, Bad Homburg, Germany). Animals were observed for two dark–light cycles after an initial accommodation phase of several hours (Wittman et al., 2008).

9.11 STATISTICAL ANALYSIS OF BEHAVIORAL EXPERIMENTS

For comparison of control groups and actein-treated groups the Student’s t-test was used. Comparison of more than two groups was done by one-way ANOVA, applying GraphPad Prism 4.0 software. P-values of < 0.05 were accepted as statistically significant. All data are given as mean ± SEM (n).

10. RESULTS

The effects of *Valerian* and *Black Cohosh* extracts and selected natural products from these plants on $GABA_A$ receptor function were investigated. For this purpose, $GABA_A$ receptors comprising different subunit compositions were expressed in *Xenopus* oocytes and the modulation of GABA-induced currents (I_{GABA}) investigated by means of the 2-microelectrode voltage clamp technique (TEVC) and a fast perfusion system. To analyse the potentially sedative, hypnotic and anxiolytic effects of actein *in vivo* the Open-field-, the Dark-Light Choice, the Elevated Plus Maze- and Stress-Induced-Hyperthermia test were applied.

10.1 VALERENIC ACID POTENTIATES AND INHIBITS $GABA_A$ RECEPTORS: MOLECULAR MECHANISM AND SUBUNIT SPECIFITY

10.1.1 MODULATION OF I_{GABA} BY *VALERIAN* EXTRACTS

The modulation of I_{GABA} by commercially available Valerian extracts (kindly provided by Univ. Prof. DDr. Brigitte Kopp and colleagues) was investigated on recombinant $GABA_A$ receptors ($\alpha_1\beta_2\gamma_{2S}$) expressed in *Xenopus laevis* oocytes. As illustrated in Fig. 17, the extracts at a concentration of 100 µg/ml significantly stimulated ($p<0.05$) I_{GABA}. The strongest effect was observed for extracts C3 and C4, enhancing I_{GABA} by 133.9 ± 15.9% (n=6) and 123.4 ± 21.0% (n=6), respectively. The effect was less pronounced for the extracts C1 (39.7 ± 8.8 %, n=7), C2 (24.0 ± 5.8 %, n=6) and C5 (23.5 ± 11.7%, n=4).

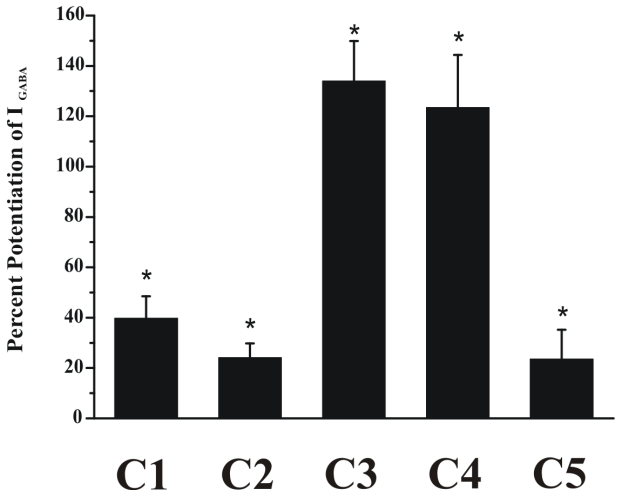


Figure 17 Modulation of I_{GABA} through $GABA_A$ receptors composed of $\alpha_1\beta_2\gamma_{2S}$-subunits by *Valerian* extracts of different polarity at a concentration of 100 µg/ml. Each value represents the mean ± S.E.

from at least 4 oocytes and ≥ 2 oocyte batches. (*) indicates significantly different from zero ($p < 0.05$).

10.1.2 POTENTIATION OF I_{GABA} BY VALERENIC ACID THROUGH $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_1$ AND $\alpha_1\beta_2\gamma_{2S}$ CHANNELS

The modulation of I_{GABA} by VA was investigated on recombinant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. Modulation of I_{GABA} by valerenic acid was first studied on $GABA_A$ channels composed of either $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_{1/2S}$ subunits. As shown in Fig. 19 A, B valerenic acid exhibited a positive allosteric modulatory effect at concentrations ≥1 µM by enhancing I_{GABA}. The effect was dose-dependent and the averaged concentration-response curve shows that maximum stimulation of $\alpha_1\beta_2\gamma_{2S}$ receptors occurred at ~100 µM (Fig. 19 B). The maximal potentiation of I_{GABA} (efficiency) through $\alpha_1\beta_2\gamma_1$ **(**235.6 ± 46.4%, n=8) and $\alpha_1\beta_2\gamma_{2S}$ channels (400.0 ± 77.6 %, n=12) was however not significantly higher than in channels composed of α_1 and β_2 subunits **(**245.7 ± 53.3 %, n=17) (see Fig. 19 A and B, Tables 1 and 2). At concentrations ≥30 µM a direct activation of $GABA_A$ channels by valerenic acid was observed. This finding is illustrated in the inset of Fig. 18 A (see Fig. 23 for detailed analysis of channel activation by valerenic acid). At concentrations ≥100 µM current enhancement by valerenic acid was less pronounced or even inhibition of control I_{GABA} was evident (Khom et al., 2007).

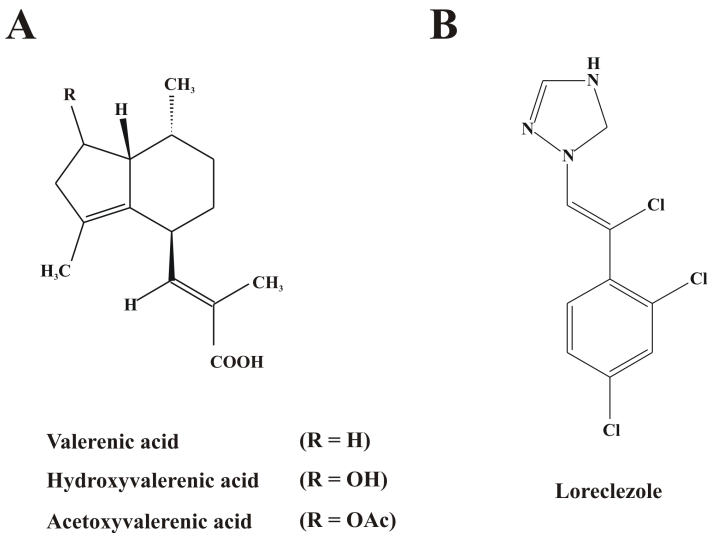


Figure 18: (A) Structure of valerenic acid and derivatives **(B)** chemical structure of loreclezole (Khom et al., 2007)

10.1.3 POTENTIATION OF I_{GABA} BY VALERENIC ACID THROUGH $\alpha_2\beta_2$, $\alpha_3\beta_2$, $\alpha_4\beta_2\gamma_{2S}$, $\alpha_5\beta_2$, $\alpha_2\beta_2\gamma_1$ AND $\alpha_2\beta_2\gamma_{2S}$ CHANNELS

A possible α-subunit specificity was analysed by substituting the α_1 by α_2, α_3, α_4 and α_5 subunits and subsequent analysis of the modulation of I_{GABA} through the corresponding $GABA_A$ channels. A comparison of Fig. 19 B and C reveals a very similar modulation of $GABA_A$ receptors incorporating α_2 instead of α_1 subunits. As illustrated in Fig. 19 B and C a slightly higher efficiency of valerenic acid in γ_{2S}-containing receptors was observed (see Table 1 for fitted efficiencies and EC_{50}s**).** A reliable estimation of the EC_{50} values and maximal I_{GABA} stimulation was complicated by an apparent I_{GABA} inhibition at concentrations ≥100 µM that was consistently observed for all subunit compositions (except for $\alpha_2\beta_2\gamma_1$ receptors). The bell-shaped concentration-effect curves might reflect a low-affinity open-channel block at high valerenic acid concentrations (see Fig. 24). Maximal stimulation of I_{GABA} through $GABA_A$ receptors composed of $\alpha_3\beta_2$ and $\alpha_5\beta_2$ subunits was comparable to $\alpha_2\beta_2$ or $\alpha_1\beta_2$ receptors (see Table 1 for efficiencies and EC_{50} values, compare Fig.19 B, C and D and Table 2). Interestingly, $\alpha_4\beta_2\gamma_{2S}$ receptors displayed a significantly lower sensitivity for valerenic acid with a maximal enhancement of I_{GABA} of 68.9 ± 14.1% (Khom et al., 2007).

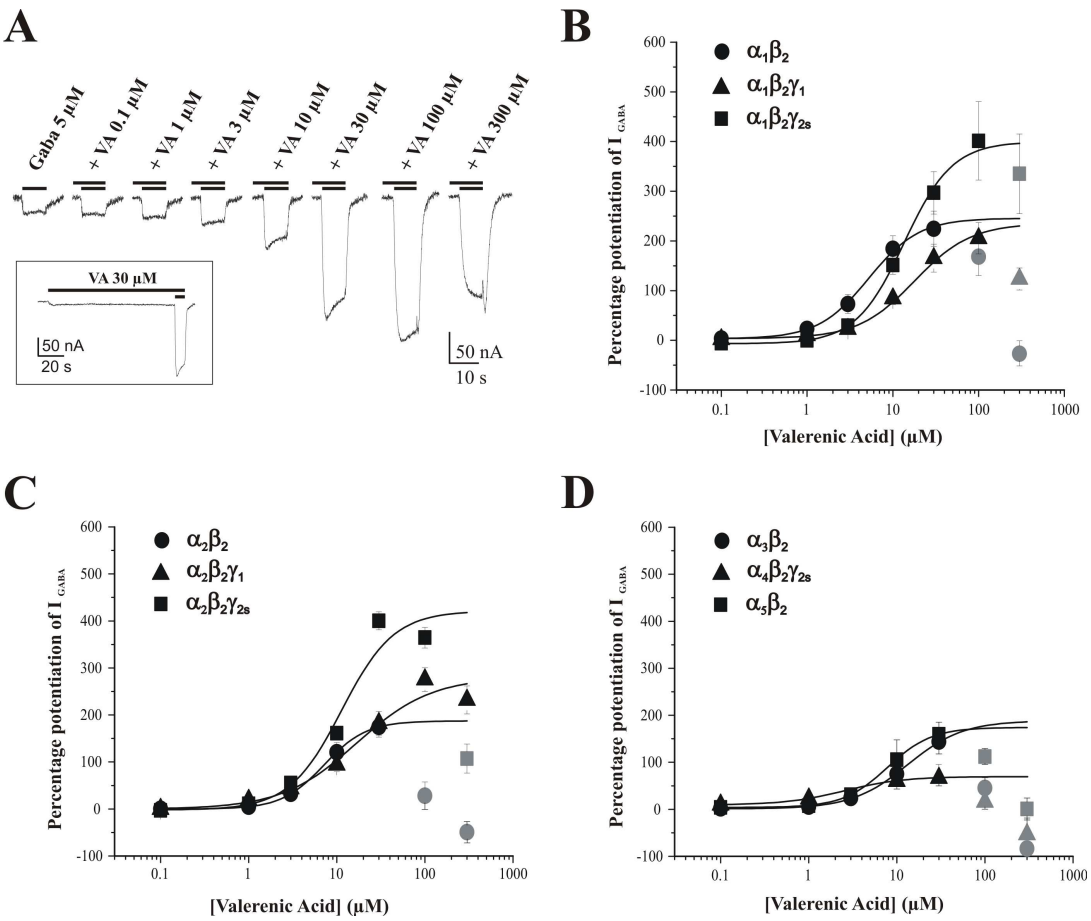


Figure 19 (A) Typical I_{GABA} recordings illustrating concentration-dependent modulation by VA of GABA elicited chloride currents through $\alpha_1\beta_2\gamma_{2S}$-containing receptors. Direct activation of $GABA_A$ receptors during preincubation with 30 µM VA is shown in an inset. Concentration-effect curves for action of VA on **(B)**: $\alpha_1\beta_2$ (●), $\alpha_1\beta_2\gamma_1$ (▲) and $\alpha_1\beta_2\gamma_{2S}$ (■), **(C)**: $\alpha_2\beta_2$ (●), $\alpha_2\beta_2\gamma_1$ (▲) and $\alpha_2\beta_2\gamma_{2S}$ (■) and **(D)**: $\alpha_3\beta_2$ (●), $\alpha_4\beta_2\gamma_{2S}$ (▲) and $\alpha_5\beta_2$ (■) receptors using an GABA $EC_{5\text{-}10}$ (EC_{50} and n_H values are given in Table 1). Data points represent means ± S.E. from at least 4 oocytes from ≥ 2 batches. I_{GABA} at 100 and 300µM ($\alpha_1\beta_2$) and at 300 µM ($\alpha_1\beta_2\gamma_{2S}$ and $\alpha_1\beta_2\gamma_1$) (Fig. B) (grey symbols), I_{GABA} at 100 and 300 µM ($\alpha_2\beta_2$) and at 300 µM ($\alpha_2\beta_2\gamma_{2S}$) (Fig. C) (grey symbols) and I_{GABA} at 100 µM and 300 µM ($\alpha_3\beta_2$, $\alpha_4\beta_2\gamma_{2S}$ and $\alpha_5\beta_2$) (Fig. D) (grey symbols) were excluded from the fit (Khom et al., 2007).

Subunit combination	EC_{50} (μM)	Maximum stimulation of I_{GABA} (EC_{5-10}) (%)	Hill-Coefficient (n_H)	Number (n) of experiments
$\alpha_1\beta_2$	5.2 ± 2.4	245.7 ± 53.3	1.5 ± 0.4	n = 5
$\alpha_1\beta_3$	16.6 ± 3.8	487.3 ± 78.9	1.7 ± 0.3	n = 5
$\alpha_1\beta_2\gamma_1$	18.0 ± 7.1	235.6 ± 46.4	1.3 ± 0.2	n = 4
$\alpha_1\beta_2\gamma_{2S}$	13.6 ± 4.1	400.0 ± 77.6	1.5 ± 0.3	n = 4
$\alpha_2\beta_2$	7.2 ± 2.6	187.4 ± 36.8	1.8 ± 0.6	n = 5
$\alpha_2\beta_2\gamma_1$	16.8 ± 6.4	280.5 ± 36.1	1.0 ± 0.2	n = 4
$\alpha_2\beta_2\gamma_{2S}$	11.3 ± 1.6	421.4 ± 26.3	1.5 ± 0.1	n = 4
$\alpha_3\beta_2$	13.2 ± 10.5	188.5 ± 90.4	1.4 ± 0.5	n = 5
$\alpha_5\beta_2$	7.8 ± 6.1	174.2 ± 57.0	1.7 ± 1.1	n = 4
$\alpha_4\beta_2\gamma_{2S}$	2.3 ± 4.6	69.0 ± 23.6	1.5 ± 1.6	n = 4
$\alpha_1\beta_{1S290N}$	10.4 ± 3.8	371.3 ± 69.6	1.5 ± 0.3	n = 4

Table 1 Potency and efficiency of Valerenic acid for $GABA_A$-receptors of different subunit compositions.

I_{MAX} / EC_{50}	$\alpha_1\beta_2$	$\alpha_1\beta_3$	$\alpha_1\beta_2\gamma_1$	$\alpha_1\beta_2\gamma_{2S}$	$\alpha_2\beta_2$	$\alpha_2\beta_2\gamma_1$	$\alpha_2\beta_2\gamma_{2S}$	$\alpha_3\beta_2$	$\alpha_5\beta_2$	$\alpha_4\beta_2\gamma_{2S}$	$\alpha_1\beta_{1S290N}$
$\alpha_1\beta_2$		*					*			*	
$\alpha_1\beta_3$	*		*		*	*		*	*	*	
$\alpha_1\beta_2\gamma_1$							*			*	
$\alpha_1\beta_2\gamma_{2S}$					*				*	*	
$\alpha_2\beta_2$		*					*			*	*
$\alpha_2\beta_2\gamma_1$							*			*	
$\alpha_2\beta_2\gamma_{2S}$	*							*	*	*	
$\alpha_3\beta_2$											
$\alpha_5\beta_2$											*
$\alpha_4\beta_2\gamma_{2S}$		*	*	*		*	*				*
$\alpha_1\beta_{1S290N}$											

Table 2 Comparison of efficiencies (upper-right) and potencies (lower-left) for $GABA_A$ receptors of different subunit compositions. (*) indicates statistically significant ($p<0.05$) differences.

10.1.4 MODULATION OF I_{GABA} BY VALERENIC ACID AT DIFFERENT GABA CONCENTRATIONS

In order to gain insight into the mechanism of I_{GABA} modulation by valerenic acid, GABA concentration-response curves in the presence and absence of valerenic acid on $\alpha_1\beta_3$ channels were compared. Valerenic acid was applied in a concentration of 30 µM to minimise a potential inhibition of I_{GABA} (see Fig. 19 B, C and D). At this concentration valerenic acid shifted the concentration-effect curve towards lower GABA concentrations and also slightly increased the maximal GABA response (Fig. 20) (Khom et al., 2007).

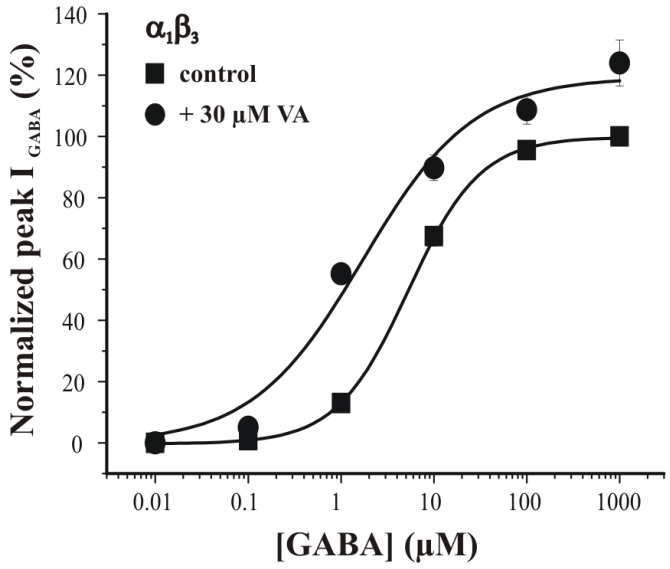


Figure 20 GABA concentration-effect curve of $\alpha_1\beta_3$ $GABA_A$ receptors (control, ■) and in the presence of 30 µM VA (●). Corresponding EC_{50}-values were 5.3 ±0.6 µM (n_H= 1.1 ± 0.1, n=4) for control and 1.7 µM ± 0.7 µM (n_H =0 .7 ± 0.2, n=4) in the presence of VA, respectively. The increase in the maximal I_{GABA} may result from a direct activation of $GABA_A$ receptors (see Fig. 23). Data points represent means ± S.E. from at least 4 oocytes from ≥ 2 batches (Khom et al., 2007).

10.1.5 VALERENIC ACID DOES NOT INTERACT WITH THE BZD SITE

Valerenic acid has a strong stimulatory effect on $GABA_A$ channels not containing γ subunits. A trend towards a slightly higher efficiency of valerenic acid on $\alpha_{1/2}\beta_2$ receptors containing a γ_{2S} subunit (Fig. 19 B-D), however, suggested a possible interaction of valerenic acid with the benzodiazepine binding pocket. To investigate this, I_{GABA} through $\alpha_1\beta_2\gamma_2$ receptors was stimulated with 5 µM valerenic acid either in the absence or presence of 1 µM of the benzodiazepine antagonist flumazenil. Flumazenil did not affect I_{GABA} potentiation significantly (77.4 ± 2.3 %, n=3 control vs. 77.3 ± 8.6%, n=3) (Fig. 21 A, B). Moreover,

additive effects of valerenic acid and 300 nM diazepam on $\alpha_1\beta_2\gamma_{2S}$ receptors also suggest that the binding sites of valerenic acid and the BZD like diazepam are independent of each other (Fig. 21 C, D) (Khom et al., 2007).

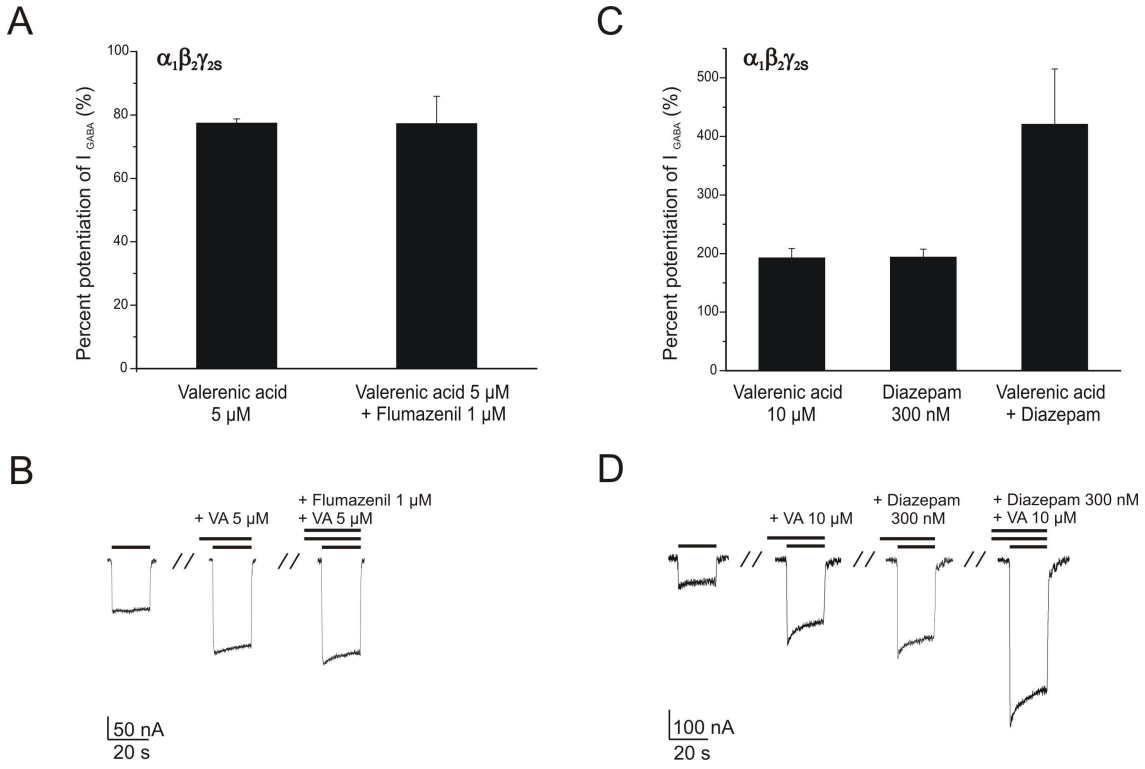


Figure 21 (A) Stimulation of I_{GABA} by 5 µM VA is not inhibited by flumazenil. The left bar shows the positive allosteric modulation of the GABA (EC_{5-10})-induced chloride current by 5 µM VA through $\alpha_1\beta_2\gamma_{2S}$ receptors. The right bar illustrates that flumazenil (1 µM) does not antagonize VA-induced enhancement of I_{GABA}. **(B)** Typical I_{GABA} through $\alpha_1\beta_2\gamma_{2S}$ receptors in the absence and presence of the indicated concentrations of VA or VA and flumazenil, respectively. **(C)** Additive effects of VA 10 µM and diazepam 300 nM. The left bar illustrates the enhancement of I_{GABA} by 10 µM VA, the bar in the middle by 300 nM diazepam, whereas the right bar illustrates the enhancement of I_{GABA} under coapplication of VA 10 µM and diazepam 300 nM through receptors composed of α_1, β_2 and γ_{2S} subunits. **(D**) Representative currents through $\alpha_1\beta_2\gamma_{2S}$ channels for the additive effects of VA and diazepam (Khom et al., 2007).

10.1.6 β-SUBUNIT DEPENDENCE OF $GABA_A$ RECEPTOR POTENTIATION BY VALERENIC ACID

A strong dependence of the modulatory effect of valerenic acid on the β-subunit composition of the receptor was observed. Fig. 22 illustrates the effect of valerenic acid on I_{GABA} through

$GABA_A$ channels with different β-subunit isoforms (channels without γ-subunits). As illustrated in Fig. 22 only channels incorporating β_2 or β_3 subunits were stimulated by valerenic acid at concentrations between 1 and 30 µM. The highest efficiency of valerenic acid was observed for $\alpha_1\beta_3$ channels. Replacing $\beta_{2/3}$ by β_1 drastically reduced the sensitivity of the resulting $GABA_A$ channels. As shown in Fig. 22 A, the stimulation of I_{GABA} through $\alpha_1\beta_1$ receptors was almost absent. Interestingly, the point mutation β_{2N265S} (known to reduce stimulatory effects by loreclezole, Wingrove et al., 1994) almost completely abolished the stimulatory effect of valerenic acid. The corresponding mutation in β_1 (β_{1S290N}), however, induced sensitivity for valerenic acid. These findings suggest an involvement of this residue in either binding of valerenic acid or transduction of the effect. Corresponding representative I_{GABA} in absence and presence of 10 µM valerenic acid are shown in Fig. 22 B. The structures of valerenic acid, acetoxy- valerenic acid, hydroxy- valerenic acid and loreclezole are illustrated in Fig. 18 (Khom et al., 2007).

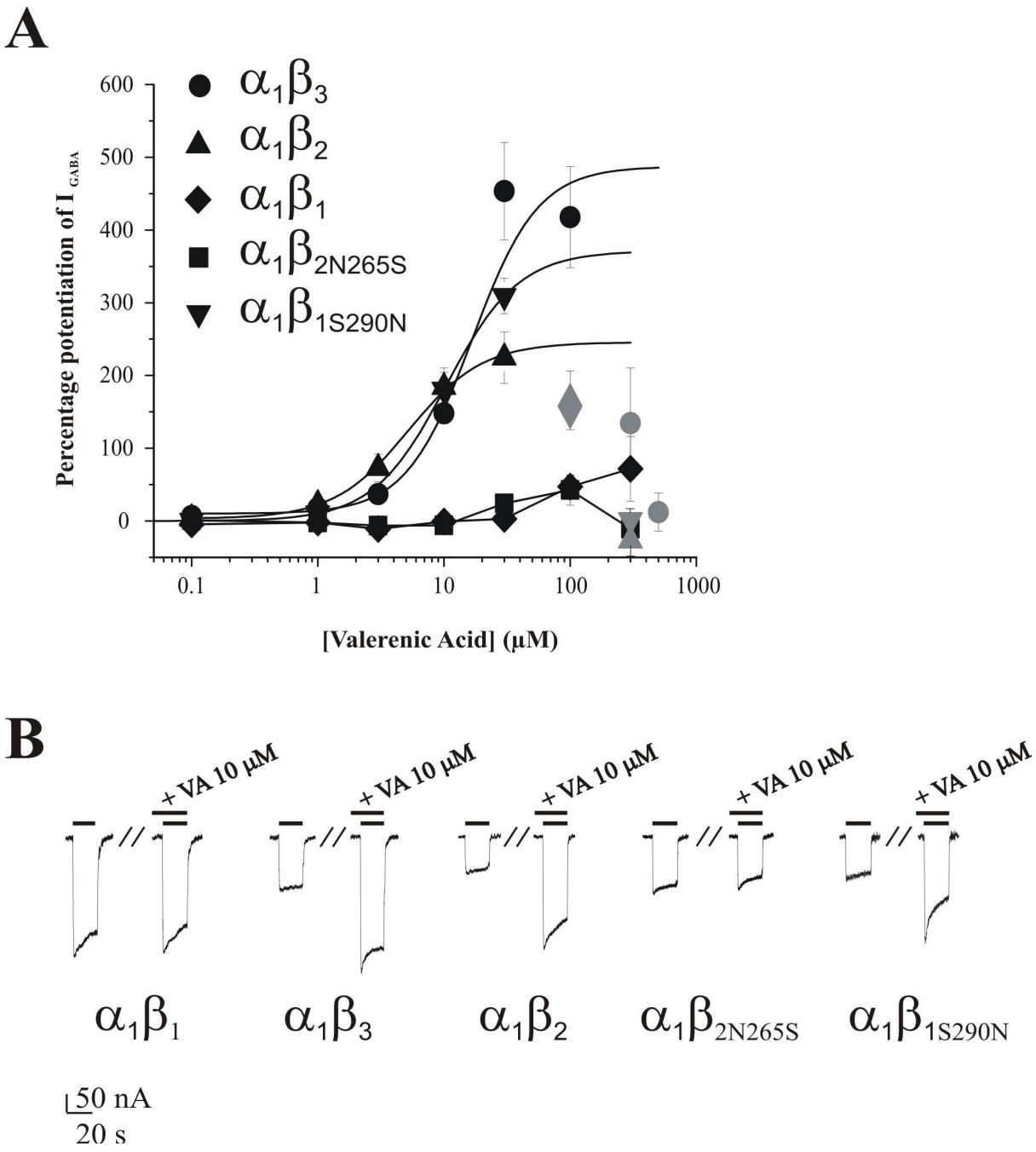


Figure 22 (A) Concentration-dependent effect for VA on $\alpha_1\beta_1$, $\alpha_1\beta_3$, $\alpha_1\beta_2$, $\alpha_1\beta_{2N265S}$ and $\alpha_1\beta_{1S290N}$ receptors using a GABA EC_{5-10}. **(B)** Typical traces for modulation of chloride currents through $\alpha_1\beta_1$, $\alpha_1\beta_3$, $\alpha_1\beta_2$, $\alpha_1\beta_{2N265S}$ and $\alpha_1\beta_{1S290N}$ channels by 10μM valerenic acid at GABA EC_{5-10}. Control currents (GABA, single bar) and corresponding currents elicited by coapplication of GABA and the indicated valerenic acid concentration (double bar) are shown (Khom et al., 2007).

10.1.7 DIRECT ACTIVATION OF $GABA_A$ RECEPTORS BY VALERENIC ACID

As shown in Fig. 19 A, valerenic acid induced currents through $GABA_A$ receptors (I_{VA}). Fig. 23 A illustrates valerenic acid-evoked currents through channels composed of α_1 and β_2 subunits. A comparison with the kinetics of an I_{GABA} at low GABA concentrations revealed a significantly slower activation of I_{VA} (Fig. 23 A). Compared to a mean current rise time ($t_{10-90\%}$) of I_{GABA} at EC_{10} (180 ± 45 ms, n=7) a significantly slower activation of I_{VA} with mean $t_{10-90\%}$ of 680 ± 50 ms (n=5) at valerenic acid concentrations between 30 to 300 µM was oberserved. Interestingly, not only the activation of $GABA_A$ channels but also the deactivation rate of I_{VA} were remarkably slower compared to I_{GABA}. The mean time-constants of I_{VA} deactivation of 2083 ± 232 ms (n=5 at 30 - 300 µM VA) were about 10 times larger compared to I_{GABA} decay upon washout (200 ± 10 ms, GABA EC_{10}, n=7, Fig. 23 A). With higher valerenic acid concentrations, a "wash-off" current (WOC) was observed during rapid perfusion of the oocytes with control solution (see Fig. 23 A and C). This finding suggests rapid unbinding of valerenic acid from a (low affinity) binding site located in the open channel pore (compare with Akk and Steinbach, 2000; Feng et al., 2004). The amplitude of the "wash-off" current increased with increasing valerenic acid concentration from 30 to 300 µM (Fig. 23) (Khom et al., 2007).

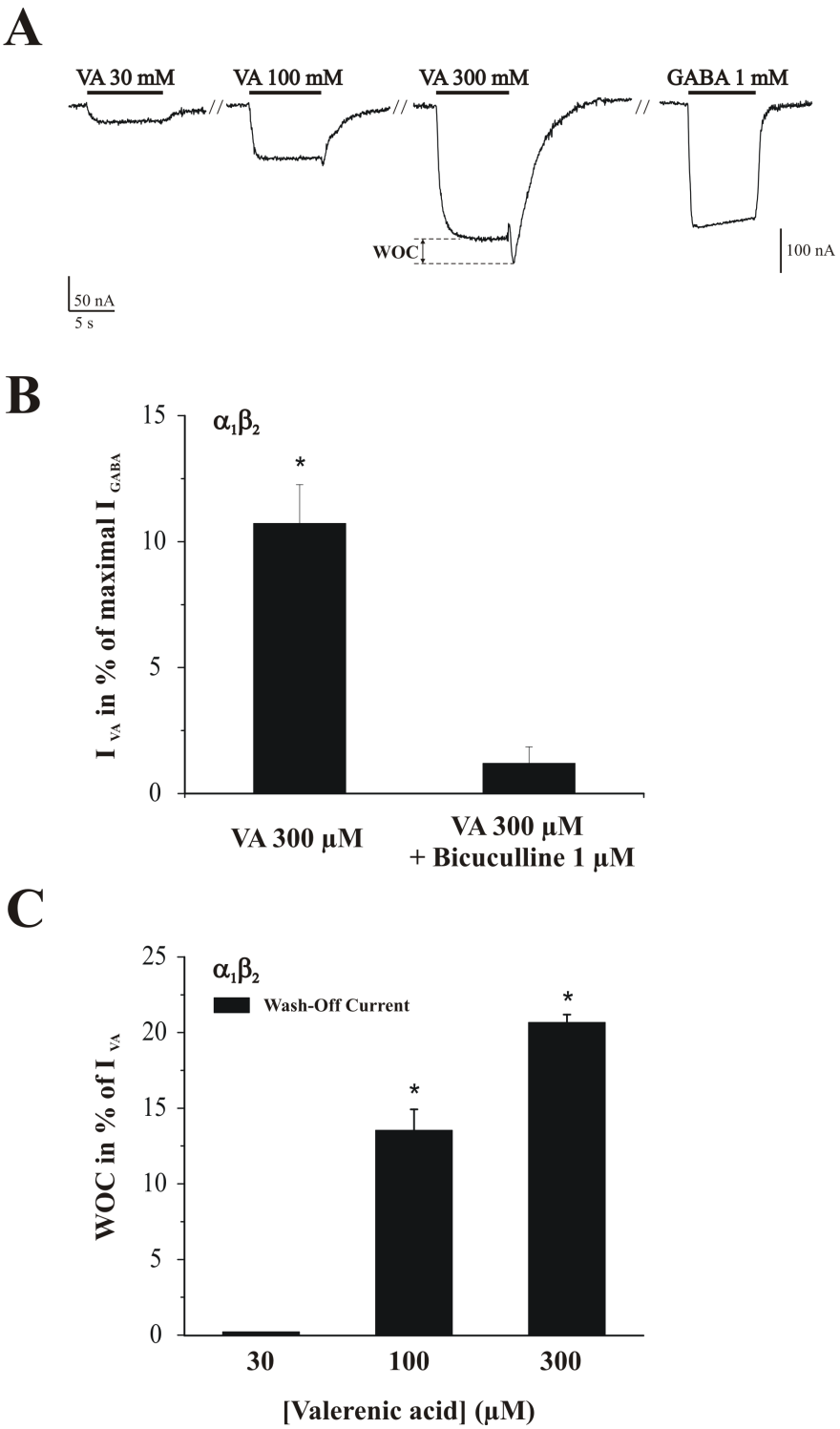


Figure 23

(A) Representative currents illustrating direct activation of $GABA_A$ receptors ($\alpha_1\beta_2$) by VA at the indicated concentrations in comparison to a GABA-induced current at EC_{10}. **(B)** Inhibition of I_{VA} (300 µM) by bicuculline. The left bar illustrates the percentage of the VA-induced current (300 µM) in relation to the maximal I_{GABA} at 1mM. The right bar shows the effect of VA in the presence of 5 µM bicuculline. (*) indicates statistically significant differences from zero ($p < 0.05$).

(C) Estimation of the relative "wash-off" current (WOC) in percentage of corresponding I_{VA} at the indicated VA concentrations (Khom et al., 2007).

10.1.8 EVIDENCE FOR OPEN CHANNEL BLOCK BY VALERENIC ACID AND ACETOXY- VALERENIC ACID

The data shown in Fig. 23 suggest an open channel block mechanism for valerenic acid. This hypothesis is supported by the observed “wash-off” currents that increased at high valerenic acid concentrations (Fig. 23). Interestingly, acetoxy- valerenic acid induced no stimulation at concentrations up to 100 µM (and did not evoke currents when applied at this concentration alone, data not shown) but accelerated the current decay through $\alpha_1\beta_3$ channels (Fig. 24 A). Making use of these properties (no stimulation but acceleration of current decay) inhibition of I_{GABA} in more detail could be studied. The shape of I_{GABA} in the presence of 100 and 300 µM acetoxy- valerenic acid revealed a concentration dependent acceleration of the current decay (Fig. 24 B), which would agree with an open-channel block. Interestingly, only acetoxy- but not hydroxy- valerenic acid accelerated the I_{GABA} decay (data not shown). Plotting the reciprocal of the time constants of the acetoxy- valerenic acid induced current decay against concentration enabled the estimation of an apparent IC_{50} value (~190 µM) for an apparent open channel block mechanism. A simulation of the bell-shaped concentration-response curve of valerenic acid action on $\alpha_1\beta_3$ channels assuming a low affinity open channel block mechanism is shown latter (see Fig. 25) (Khom et al., 2007).

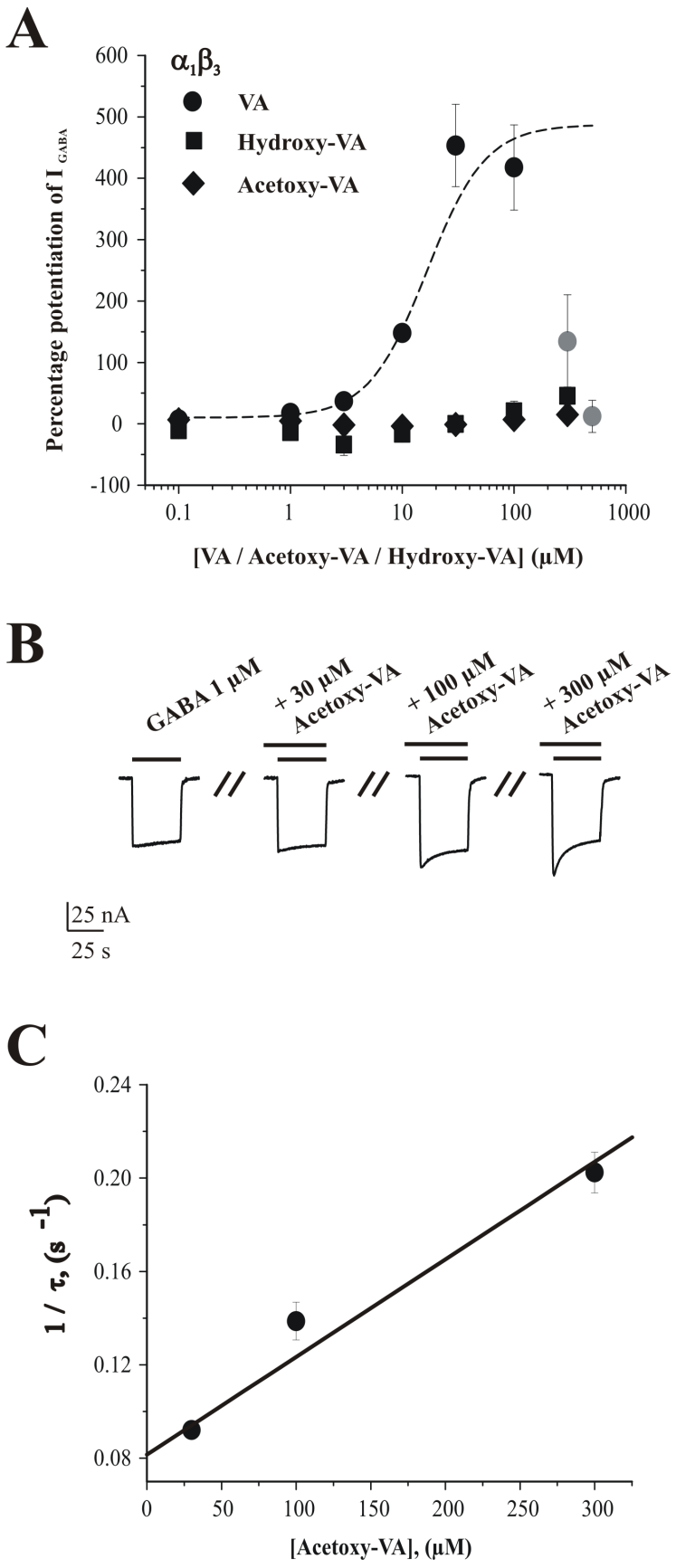


Figure 24 (A) Concentration-dependent effects for acetoxy-VA (♦), hydroxy-VA (■) on the enhancement of I_{GABA} (EC_{5-10}) through $\alpha_1\beta_3$ channels. Broken line illustrates the enhancement of I_{GABA} by VA (taken from **Fig. 22 A**). **(B)** Representative traces illustrating GABA-induced chloride currents

through $\alpha_1\beta_3$ receptors in the absence (control) and the presence of 30 µM, 100 µM and 300 µM acetoxy-VA. In the presence of 30, 100 and 300 µM acetoxy-VA I_{GABA} decayed with time constants of 10.9 ± 0.4 s, 7.4 ± 0.5 s and 5.0 ± 0.2 s. **(C)** Reciprocals of time constants of acetoxy-VA induced I_{GABA} decay plotted as function of the acetoxy-VA concentration. The regression line yields a y-intercept (rate constant of dissociation from the open channel, $k_- = 0.08\ s^{-1}$) and a slope (rate constant of association with the open channel, $k_+ = 420\ s^{-1}M^{-1}$) suggesting an IC_{50} for open channel block of ~190 µM (Khom et al., 2007).

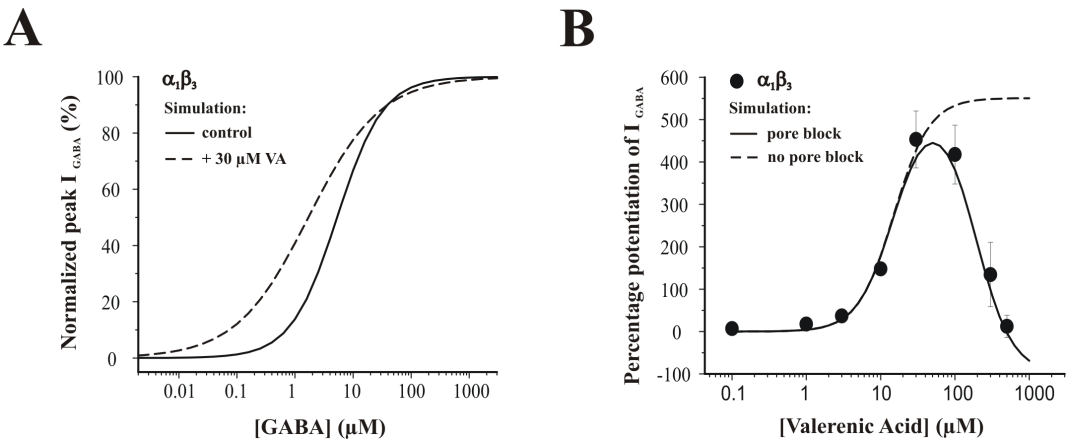


Figure 25 (A) Simulated shift of the concentration-response dependency making use of the experimental data (EC_{50}s and n_H taken from the concentration-effect curves shown in Fig. 19). The broken and straight lines illustrate the curves in the presence and absence of 30 µM VA, respectively. Direct activation of $GABA_A$ receptors by VA was not taken into account. **(B)** Simulation of concentration-dependent enhancement and inhibition of I_{GABA} by VA (in %). Solid line represents a simulation of the positive allosteric modulation by VA (EC_{50} = 15 µM, n_H = 1.8) and open channel block (IC_{50} = 190 µM, n_H = 1.8). Broken line illustrates the simulated concentration-effect curve without open channel block. The approximated IC_{50} for apparent open channel block of $GABA_A$ channels corresponds to the estimated value of acetoxy-VA (Fig. 24 C) (Khom et al., 2007).

10.2 ACTEIN- A NOVEL HIGHLY EFFICIENT MODULATOR OF $GABA_A$ RECEPTORS WITH STRONG *IN VIVO* EFFECTS

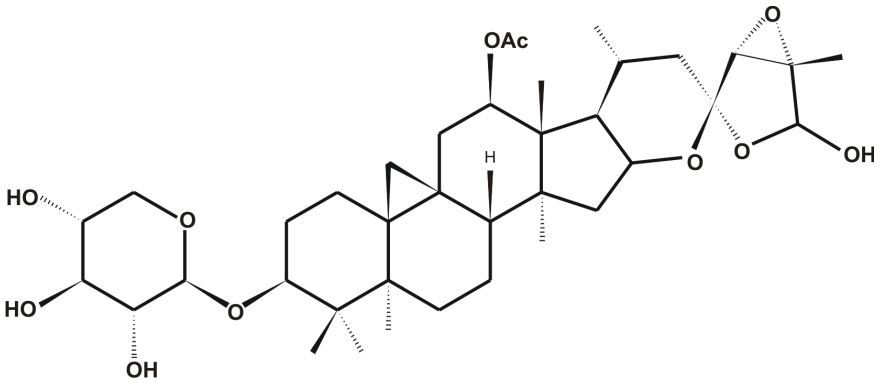


Figure 26 Chemical structure of Actein

10.2.1 MODULATION OF I_{GABA} BY *ACTAEA RACEMOSA* EXTRACTS

The modulation of I_{GABA} by *Acteae racemosa* extracts CR-I, CR-II and CR-III (kindly provided by Univ. Prof. DDr. Brigitte Kopp and colleagues) was investigated. As illustrated in Fig. 27, CR-I, CR-II and CR-III at a concentration of 100 µg/ml significantly ($p < 0.05$) enhanced I_{GABA}. CR-II and CR-III exhibited a stronger modulation of GABA-mediated currents, enhancing I_{GABA} by 130.2 ± 14.4% (n=3) and 132.8 ± 33.1% (n=3) compared to the extract CR-I, which showed 64.8 ± 4.8% potentiation of I_{GABA} (see Fig. 27).

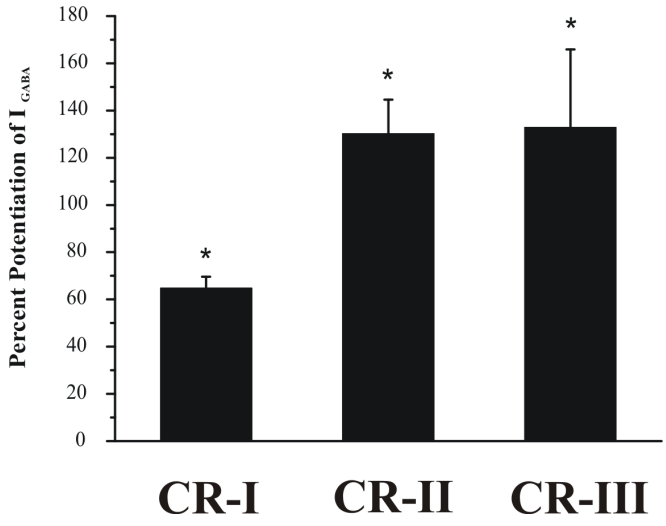


Figure 27 Enhancement of I_{GABA} by *Actaea racemosa* extracts through $GABA_A$ channels comprising $\alpha_1\beta_2\gamma_{2S}$ subunits at a concentration of 100 µg/ml at GABA EC_{5-10}. Each bar represents the mean ± S.E. from at least 3 oocytes and ≥ 2 oocyte batches. (*) indicates significantly different from zero ($p < 0.05$, *t*-test by ANOVA)

10.2.2 POTENTIATION OF I_{GABA} BY ACTEIN THROUGH $GABA_A$ CHANNELS COMPOSED OF α_1, β_2 AND γ_{2S} SUBUNITS

The effects of actein, a major constituent of *Actaea racemosa*, were examined on $GABA_A$ receptors heterologously expressed in *Xenopus laevis* oocytes. Modulation of I_{GABA} by actein was first studied on $GABA_A$ channels composed of $\alpha_1\beta_2\gamma_{2S}$ subunits. As shown in Fig. 28 A, actein exhibited a positive allosteric modulatory effect at concentrations ≥1 µM by enhancing I_{GABA} at a GABA EC_{5-10}. The effect was concentration-dependent (n=8) and the averaged concentration-response curve shows that maximum stimulation of $\alpha_1\beta_2\gamma_{2S}$ receptors occurred at ~300 µM (Fig. 28 A) and yielded 1091.2 ± 124.6% potentiation of I_{GABA} (see also Table 3). The modulatory action of actein at 100 µM was not blocked by flumazenil 1 µM (n=3, data not shown). I_{GABA} modulation was not dependent on the presence of a γ subunit. The modulation of $GABA_A$ channels composed of α_1 and β_2 subunits (n= 9) was slightly more pronounced compared to $\alpha_1\beta_2\gamma_{2S}$ receptors (see Table 3, and Fig. 28 A). At concentrations ≥ 30 µM Actein elicited small currents in the absence of GABA. This direct activation did, however, not exceed 2% of the maximal GABA-induced current (n=7) (data not shown). Representative traces for direct activation of $GABA_A$ receptors are illustrated in insets in Figs. 28 B and 29 B).

10.2.3 α-SUBUNIT DEPENDENCE OF I_{GABA} MODULATION BY ACTEIN

In order to investigate a possible α-subunit specificity of actein action, the α_1 subunit was substituted by α_2, α_3, α_4, α_5 and α_6, respectively. Different α subunits were expressed in combination with a β_2 subunit to analyse the modulation of I_{GABA} (in the case, α_4 and α_6 containing receptors additionally a γ_{2S} subunit was co-expressed) (Fig. 27 A, Table 3). The highest efficiency was observed for receptors containing either α_2 or α_3 subunits, revealing a maximal potentiation of I_{GABA} (EC_{5-10}) of 1677.6±225.3% (n=8) and 1322.5±237.8% (n= 8), respectively, followed by α_1-containing receptors (n=8).

Actein displayed a considerably lower efficiency on $GABA_A$ receptors composed of α_4 and α_6 subunits (811.6±96.6%; n=5 and 840.2±117.0%; n= 6, see also Table 3). The effect of actein was least pronounced on $GABA_A$ receptors containing α_5 subunits with an I_{GABA} enhancement of 225.8±44.8% (n=7). Comparison of the potencies of the respective subtypes revealed only minor differences, with potencies ranging from 29.5±12.9 µM ($\alpha_1\beta_2\gamma_{2S}$) to 63.5±20.0 µM ($\alpha_4\beta_2\gamma_{2S}$) (see Table 3).

Subunit combination	**EC_{50} (μM)**	**Maximum stimulation of I_{GABA} (EC_{5-10}) (%)**	**Hill- coefficient (n_H)**	**Number (n) of experiments**
$\alpha_1\beta_1$	56.2 ±12.8	2169.4±374.5	1.8±0.3	15
$\alpha_1\beta_2$	50.0±10.9	1466.7±220.7	1.6±0.3	9
$\alpha_1\beta_3$	44.5±18.2	2027.0±398.9	1.2±0.1	8
$\alpha_1\beta_2\gamma_{2S}$	29.5±12.9	1091.2±124.6	1.1±0.1	8
$\alpha_2\beta_2$	38.3±11.6	1677.6±225.3	1.6±0.3	8
$\alpha_3\beta_2$	50.8±9.3	1322.5±237.8	1.8±0.2	8
$\alpha_4\beta_2\gamma_{2S}$	63.5±20.0	811.6±94.6	1.2±0.2	5
$\alpha_5\beta_2$	43.6±22.5	225.8±44.8	1.2±0.4	7
$\alpha_6\beta_2\gamma_{2S}$	33.4±15.9	840.2±117.0	1.1±0.4	6

Table 3 Potency and efficiency of actein for $GABA_A$-receptors of different subunit compositions.

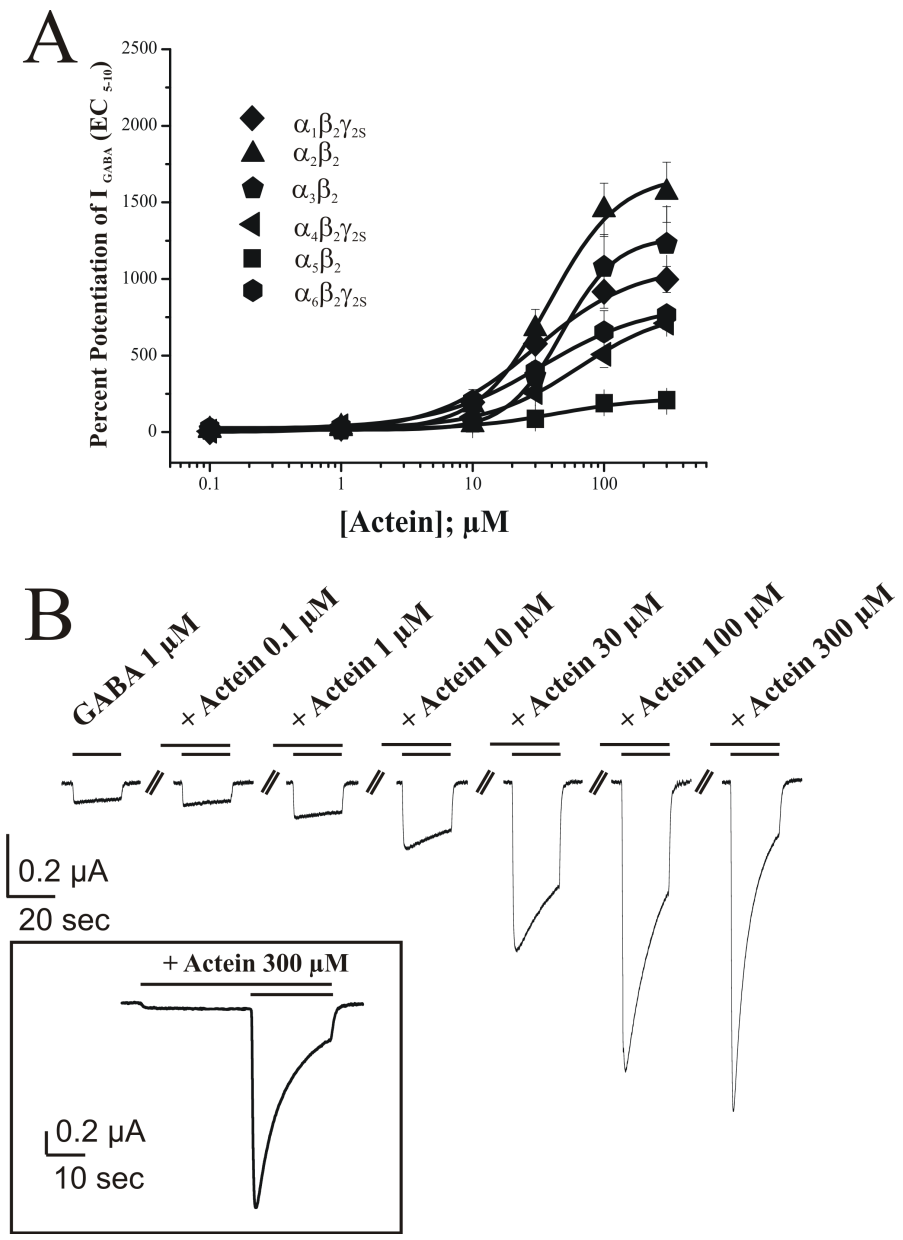


Figure 28 Modulation of I_{GABA} through $GABA_A$ receptors containing different α-subunits

(A) Concentration-effect curves for the action of actein on $\alpha_1\beta_2\gamma_{2S}$ (◆), $\alpha_2\beta_2$ (▲), $\alpha_3\beta_2$ (⬟), $\alpha_4\beta_2\gamma_{2S}$ (◄), $\alpha_5\beta_2$ (■), $\alpha_6\beta_2\gamma_{2S}$ (⬢) receptors using an EC_{5-10} GABA concentration (EC_{50} and Hill-coefficient values are given in Table 3). Data points represent means ± S.E. from at least 4 oocytes from ≥ 2 batches. **(B)** Typical I_{GABA} recordings illustrating concentration-dependent modulation by actein of GABA elicited chloride currents through $\alpha_2\beta_2$-containing receptors. Direct activation of $GABA_A$ receptors by 300 µM actein in shown in the inset.

10.2.4 INFLUENCE OF DIFFERENT β-SUBUNITS ON I_{GABA} POTENTIATION BY ACTEIN

In order to determine the contribution of the β subunits on the effect of actein on $GABA_A$ receptors, the modulation of the respective subtypes ($\alpha_1\beta_1$ and $\alpha_1\beta_3$, respectively) was analysed. A comparison of the concentration-effect curves for actein on $GABA_A$ comprising either β_1 or β_3 receptors reveals a very similar modulation of $GABA_A$ receptors in regard to both efficiency and potency (see Fig. 28, Table 3).

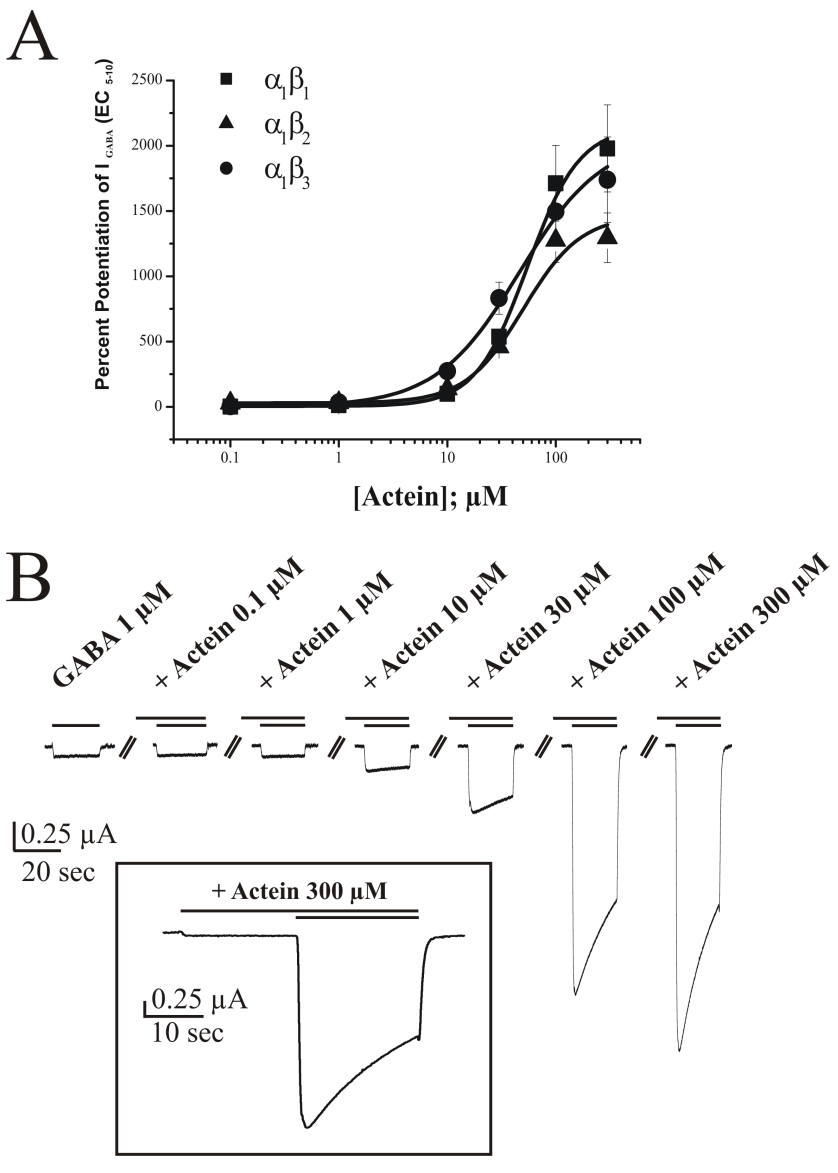


Figure 29 Influence of the β-subunit incorporated in the receptor on I_{GABA} modulation by actein
(A) Concentration-effect curves for the action of actein on $\alpha_1\beta_1$ (■)-, $\alpha_1\beta_2$ (▲)- and $\alpha_1\beta_3$ (●)-receptors using an EC_{5-10} GABA concentration (EC_{50} and Hill-coefficient values are given in Table 3). Data points represent means ± S.E. from at least 4 oocytes from ≥ 2 batches.

(B) Typical I_{GABA} recordings illustrating concentration-dependent modulation by actein of GABA elicited chloride currents through $\alpha_1\beta_1$ receptors. Direct activation of $GABA_A$ receptors during preincubation with 300 µM actein is shown in an inset.

10.2.5 MODULATION OF I_{GABA} BY ACTEIN AT DIFFERENT GABA CONCENTRATIONS

In order to gain further insight into the mechanism of I_{GABA} modulation by actein, GABA concentration-response curves in the presence and absence of actein on $\alpha_1\beta_3$ channels were compared (n=6). Actein was applied at a saturating concentration of 300 µM. At this concentration, actein shifted the concentration-effect curve towards lower GABA concentrations and also substanstially increased the maximal GABA response (Fig. 30).

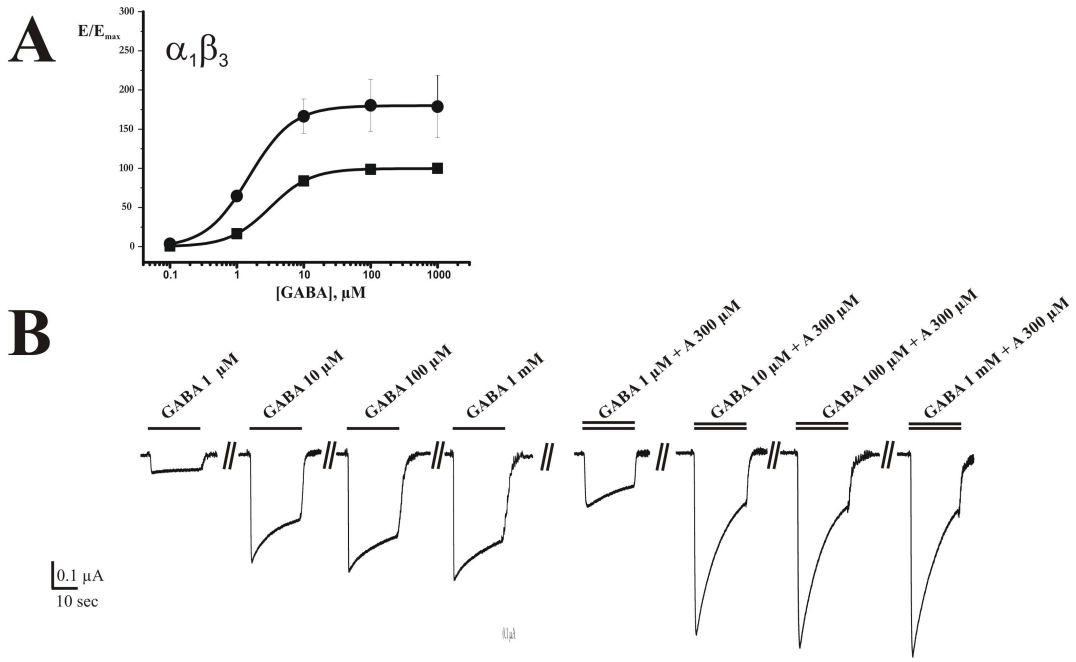


Figure 30 **(A)** GABA concentration-effect curve of $\alpha_1\beta_3$ $GABA_A$ receptors (control, ■) and in the presence of 300 µM actein (●).Corresponding EC_{50}-values and Hill-coefficients were 3.1±0.5 µM and n_H= 1.4 ±0.2 (control) and 1.5 µM ±0.3 µM and n_H =1.4 ± 0.1 (Actein), respectively.
(B) Representative traces illustrating GABA-induced chloride currents through $\alpha_1\beta_3$ receptors in the absence (control) and the presence of 300 µM actein**.**

10.2.6 PERFORMANCE IN THE OPEN FIELD TEST (OF) AFTER ACTEIN ADMINISTRATION

To determine explorative behaviour actein-treated (n=8 for all concentrations) and control (n=11) mice were studied by means of the open field test. As shown in Figure 31 A, B and C, actein at concentrations ≥2 mg/kg per kg bodyweight (KG) significantly decreased ambulation in all areas of the open field as well as the overall ambulation compared to control animals, at concentrations ≥20 mg/kg mice covered a more than 50% reduced distance (2.88±0.24m control vs. 1.35±0.16m 20 mg/kg KG actein). 0.6 mg/kg KG actein slightly increased the distance travelled in the centre region. This increase was, however, not significant. Actein (≥ 6 mg/kg) significantly ($p<0.05$) decreased the time spent in all areas including the centre (see Fig. 31 E) as well as entries into the centre (Fig. 31 D).

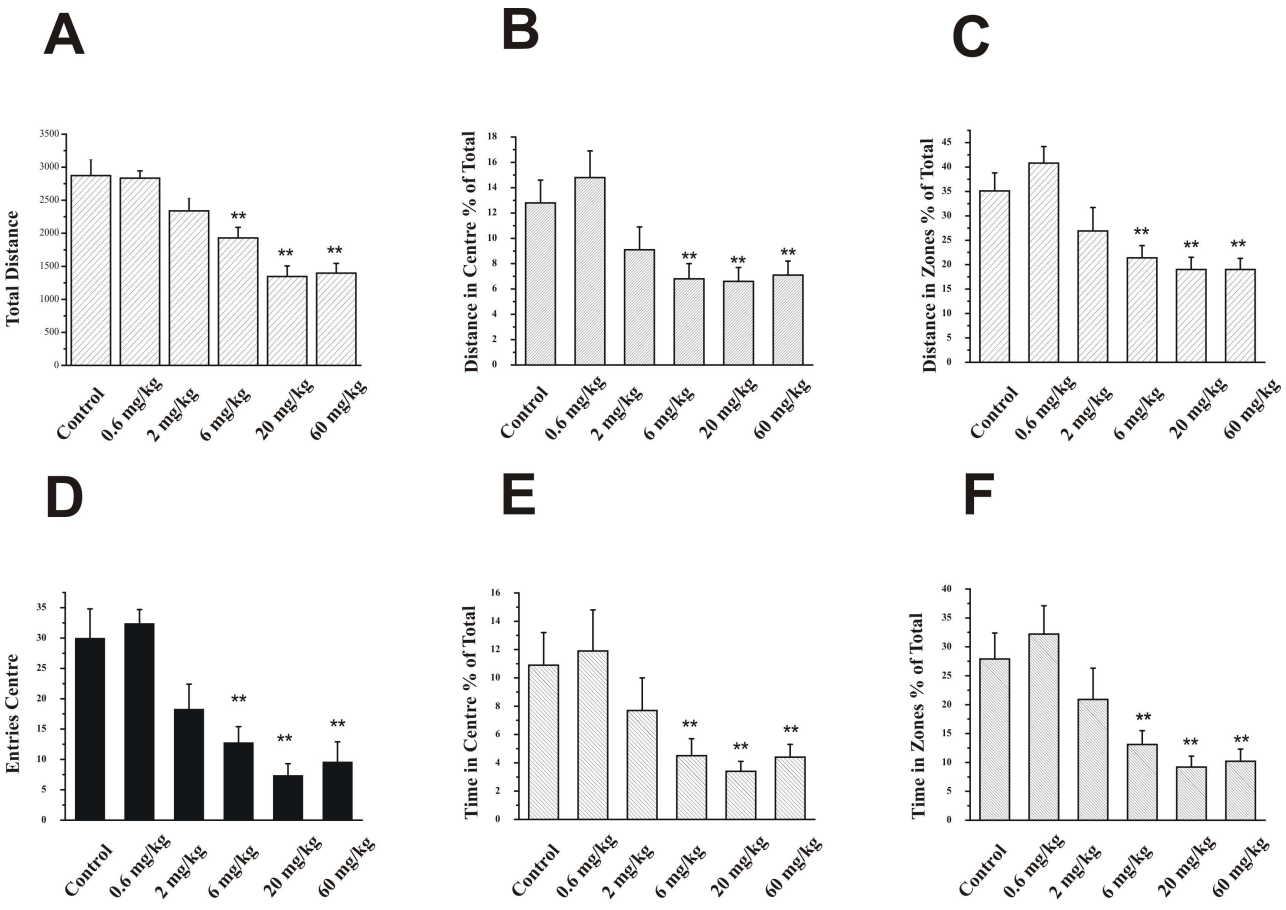


Figure 31 Ambulation and explorative behaviour in the Open Field Test was assessed over 10 min for control and actein-treated mice at the indicated concentrations (concentrations represent mg/kg KG). Bars indicate in **(A)** the total distance travelled, in **(B)** the distance travelled in centre in % of the total distance, in **(C)** the distance travelled in the centre and the intermediate region, in **(D)** the entries into the centre, in **(E)** the time spent in the centre in % of total time and in **(F)** the time spent in both the

centre and intermediate zone in % of the total time. **(**)** indicates statistically significant ($p<0.05$) differences.

10.2.7 PERFORMANCE IN THE ELEVATED PLUS MAZE (EPM) AND LIGHT-DARK CHOICE TEST (LDT) AFTER ACTEIN ADMINISTRATION

Actein significantly reduced motor activity at concentrations ≥6 mg/kg bodyweight in the open field test compared to vehicle (see Fig. 31). Thus, to assess a possible anxiolytic-like activity of actein, control mice (n=8) and their littermates treated with 0.2 (n=8) and 0.6mg/kg KG (n=8) actein were tested in the EPM. As illustrated in Fig. 32 A and B, actein at 0.2 mg /kg and 0.6 mg/kg increased the number of entries into the open arms and concurrenlty the time spent in the open arms of the elevated plus maze.

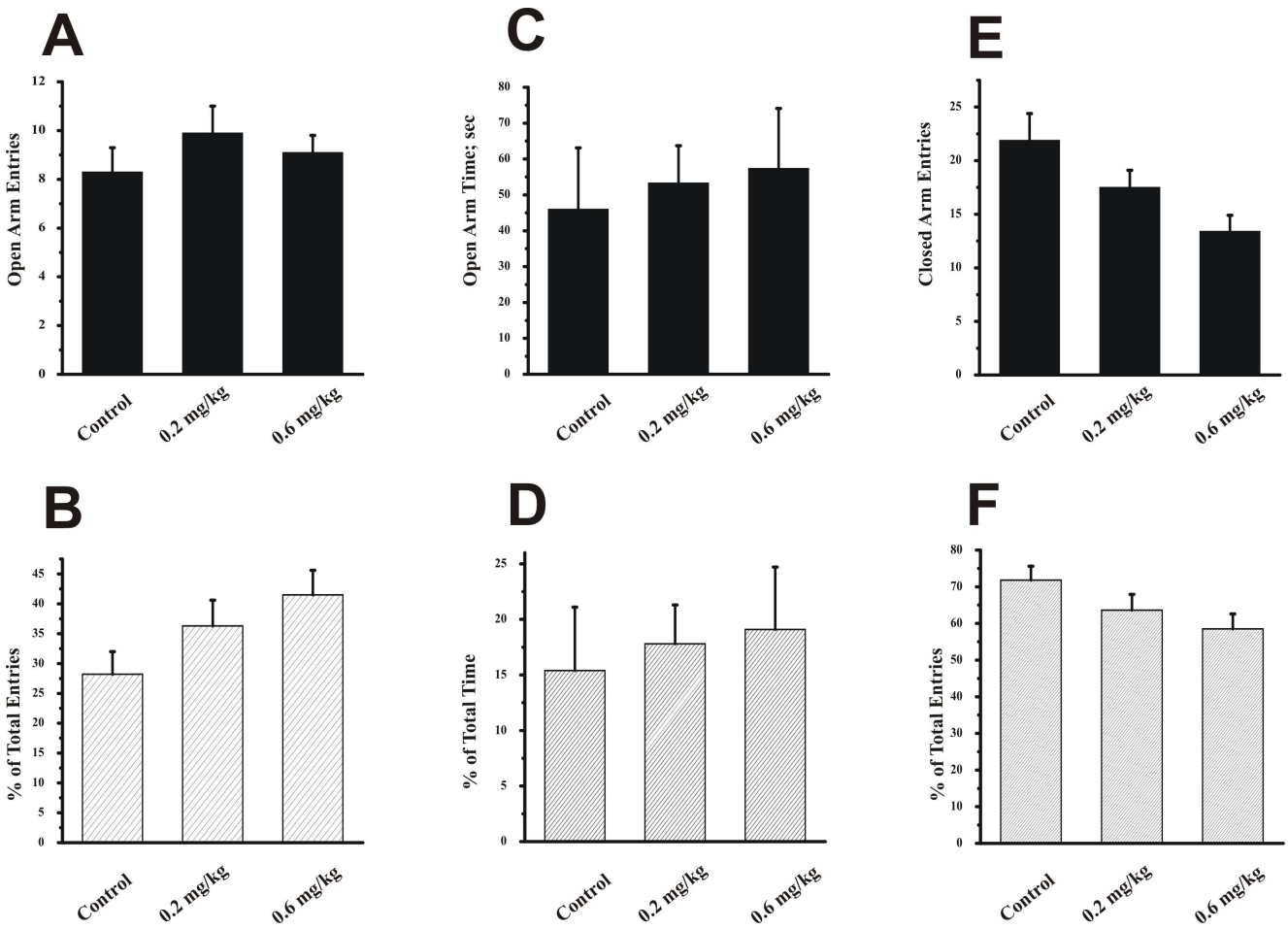


Figure 32 Behaviour in the Elevated Plus Maze Test was assessed over 5 min for control and actein-treated mice at the indicated concentrations (concentrations represent mg/kg KG). Bars indicate in **(A)** the number of open arm entries, in **(B)** the number of open arm entries in % of total entries, in **(C)** the

time spent on the open arm, in **(D)** the time spent on the open arm in % of the total time, in **(E)** the number of closed arm entries and in **(F)** the number of closed arm entries in % of the total entries.

The number of closed arm entries did not differ significantly between the groups, suggesting unaffected motor activity (see Fig. 32 E and F).

The anxiolytic effect of actein was less pronounced in the LDT. However, 0.2 mg/kg KG actein significantly increased the time spent in the lit area (Control 70.0 ± 2.5 %; n=8 vs. 0.2 mg/kg KG actein 77.1 ± 3.0%) ($p < 0.05$). Neither significant changes in distance travelled (Fig. 33 B) nor number of entries (Fig. 33 C) were observed for control and actein-treated animals at the indicated concentrations.

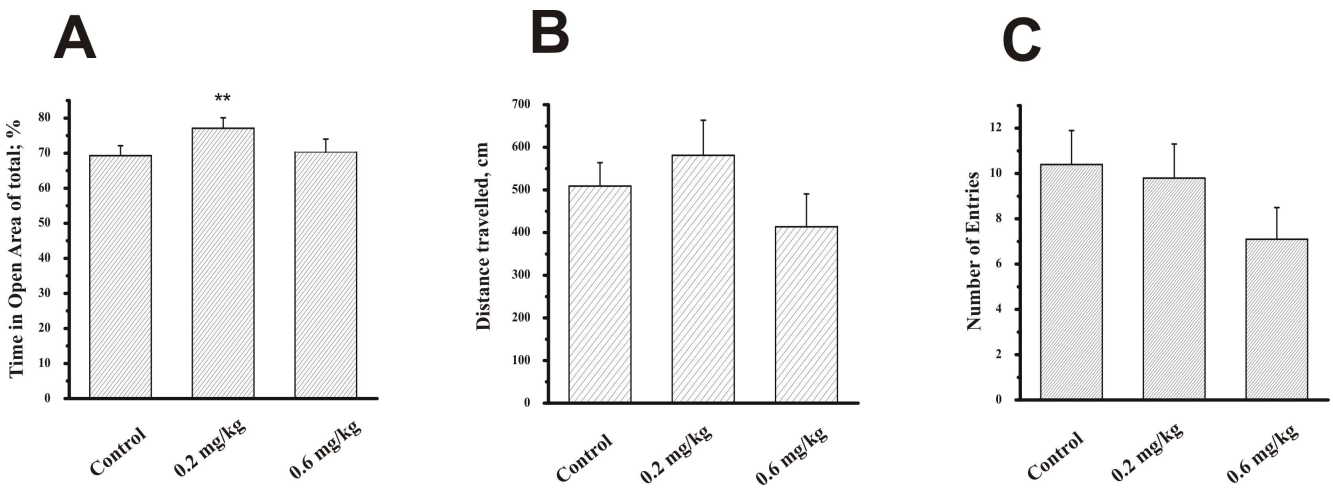


Figure 33 Behaviour in the Light-Dark Choice Test was assessed over 10 min for control and actein-treated mice at the indicated concentrations (concentrations represent mg/kg KG). Bars indicate in **(A)** the time in the lit area in % of the total time, in **(B)** the distance travelled in the lit area and in **(C)** the number entries from the dark compartment into the lit area within the first 5 min.
(**) indicates statistically significant ($p<0.05$) differences.

10.2.8 EFFECT OF ACTEIN ON STRESS-INDUCED-HYPERTHERMIA (SIH)

The basal body temperature (T1) did not differ between control mice (36.8±0.2°; n=21) and actein-treated mice at the concentratons 2 mg/kg KG (37.0±0.2°; n=9), 6 mg/kg KG (36.8 ±0.1°, n= 10) or 20 mg/kg KG (36.8 ±0.1°; n=9).

6 mg/kg KG (ΔT = 0.6 ±0.1°) and 20 mg/kg KG actein (ΔT = 0.4 ±0.1°), respectively, caused a significant reduction of ΔT ($p<0.05$) compared to vehicle (Fig. 34).

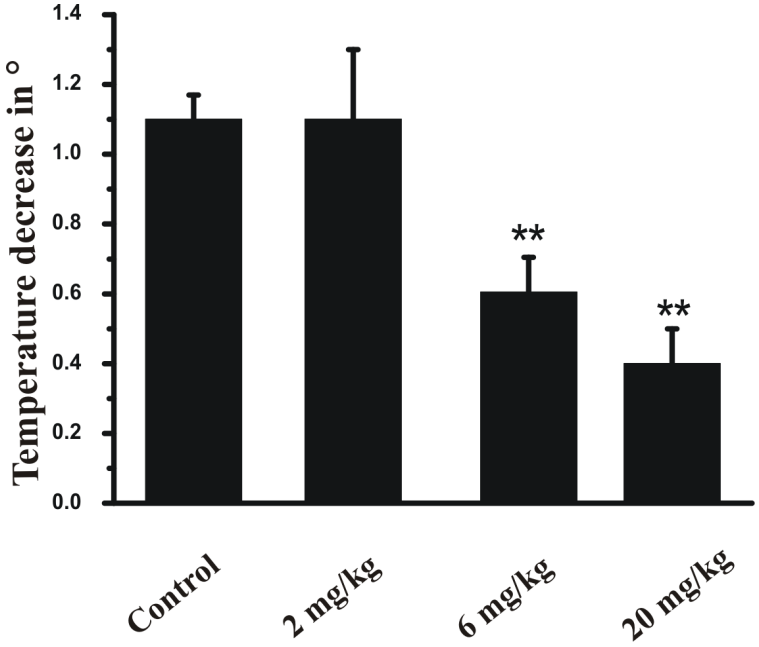


Figure 34 Effect of actein on stress-induced hyperthermia. Bars indicate reduction of temperature after actein application. **(**)** indicates statistically significant ($p<0.05$) differences.

10.2.9 HOME CAGE ACTIVITY MEASURED AFTER ACTEIN ADMINISTRATION

Basal activity and circadian rhythm of control and actein-treated mice were monitored in their home cages. Control and 2 mg/kg KG actein did not significantly differ; 6mg/kg KG actein caused a significant reduction of activity (illustrated in arbitrary mobility counts, see Fig. 35). The strongest effect was observed for 20 mg/kg KG nearly abolishing any activity. The effect reached a maximum after approximately 3 hours and lasted overall 12 hours.

After 12 hours, however, the activity of both actein-treated and control mice was indistinguishable.

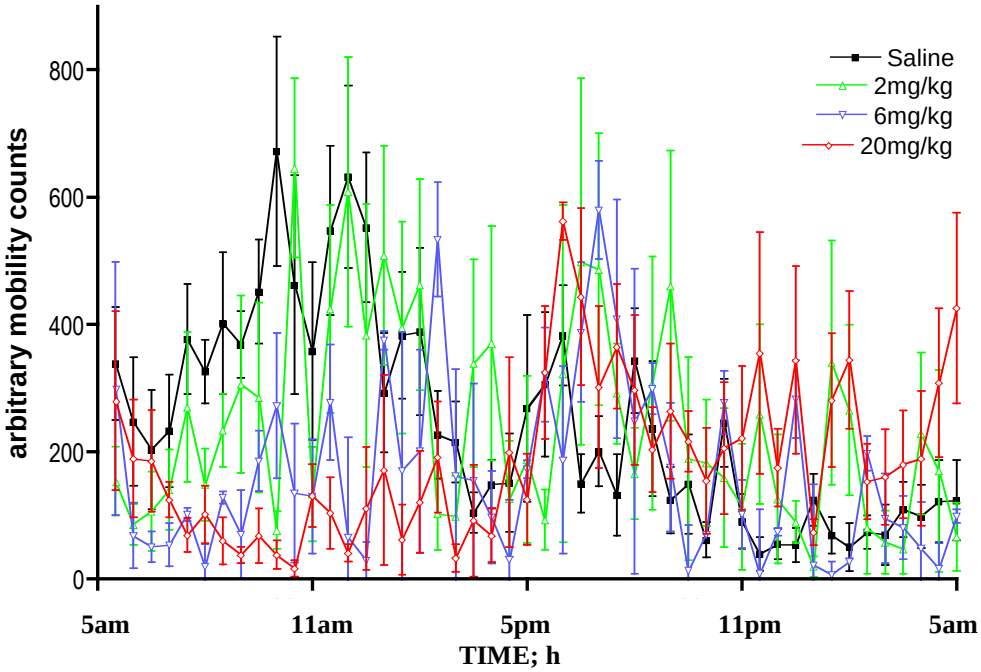


Figure 35 Effect of actein on activity in the home cage over a time period of 24 h. Black line represents the saline group=control, green line 2 mg/kg KG actein, blue line 6 mg/kg KG actein and black line 20 mg/kg, respectively; n=4 (all groups).

11. DISCUSSION

The modulation of GABA-indcued chloride currents through $GABA_A$ receptors comprising α_1, β_2 and γ_{2S} subunits by *Valerian* extracts at a concentration of 100 µg/ml was analysed by means the two-microelectrode voltage clamp technique (see Fig. 18; see also Trauner et al., 2008). As shown in Fig. 18, the extracts revealed a significant stimulation of $I_{GABA.}$ A major role of VA in I_{GABA} modulation was established in experiments, where this compound was applied at different concentrations (see Fig. 19 B, C and D) to $GABA_A$ receptors expressed in *Xenopus* oocytes.

11.1.1 SUBUNIT SPECIFICITY OF I_{GABA} STIMULATION BY VALERENIC ACID

In order to obtain insight into the molecular basis of the VA action, the modulation of $GABA_A$ channels composed of different α, β and γ subunits was analysed. As illustrated in Fig. 19, coexpression of γ_{2S} reveals a trend towards enhanced I_{GABA} stimulation (Figs. 19 B and C). This effect was, however, only significant for $\alpha_2\beta_2\gamma_{2S}$ receptors (Table 2). Omitting the γ_1 subunit did not significantly affect stimulation of either $\alpha_1\beta_2$ or $\alpha_2\beta_2$ receptors (Tables 1 and 2). The stimulatory action of VA was not significantly affected by coexpression of α_2, α_3 or α_5 instead of $\alpha_{1.}$ A lower efficiency of VA was observed for α_4 incorporating receptors ($\alpha_4\beta_2\gamma_{2S}$) (maximal enhancement of I_{GABA} 68.9 ± 14.1%), significantly different from all of the subunit compositions investigated except $\alpha_3\beta_2$ and $\alpha_5\beta_2$. This finding might help to identify determinants of VA efficiency in future studies (Fig.19 D, Tables 1 and 2).

In contrast, variation of the β subunits strongly influenced the effect on I_{GABA} stimulation by VA (Fig. 22). The highest efficiency was observed for $\alpha_1\beta_3$ followed by $\alpha_1\beta_2$ channels (Table 1). Replacing β_2 by β_1 almost completely abolished the stimulatory effect of VA. These data suggest a preferential action of VA on receptors containing β_2 or β_3 subunits. This finding was confirmed by the lack of VA action on $\alpha_1\beta_{2N265S}$ channels. Point mutation N265S in β_2 abolished the modulatory action of VA (Fig. 22). Replacing the serine in the β_1 subunit (β_{1S290}) by the corresponding asparagine of β_2 induced VA sensitivity. This finding suggests that I_{GABA} modulation may be mediated by VA interaction with the lorecelzole site, or alternatively, reflects an effect of β_{2N265S} on the transduction pathway of VA action. Similar β-subunit specificity of loreclezole action displaying strong stimulation of receptors containing either a β_2 or a β_3 subunit support this notion (Wingrove et al. 1994).

An interaction of VA with N289 at the carboxyl terminal end of the pore-forming M2 transmembrane domain could explain enhancement of I_{GABA} by lower VA concentrations (e.g. by a destabilizing the closed channel conformation) and an apparent "open channel" inhibition at higher concentrations (Khom et al., 2007).

11.1.2 SLOW ACTIVATION AND DEACTIVATION OF I_{VA}

At high concentrations, VA directly activated $GABA_A$ receptors. A direct activation of $GABA_A$ receptors was reported for a number of modulators including pentobarbital, etomidate, propofol or loreclezole (Schulz and Macdonald, 1981; Feng et al. 2004; Lam and Reynolds, 1998; Sanna et al., 1996; Hong and Wang, 2005). This VA induced current was relatively small and comparable for $\alpha_1\beta_3$ (6.2 ± 2.0%) and $\alpha_1\beta_2$ (10.7 ± 1.5%, stimulation of maximal I_{GABA}) receptors.

I_{VA} developed much slower than I_{GABA} and I_{VA} was non-desensitizing (Fig. 23). Furthermore, the current deactivation time constants ($\tau_{deact.}$) were much slower in I_{VA} compared to I_{GABA} (τ_{deact}(VA, 30-300 µM)= 2083 ± 232 ms, n=5). The time of I_{VA} deactivation thus significantly exceeds the characteristic time of solution exchange whereas the time required for washout of 1 µM GABA (τ_{deact} = 200 ± 10 ms, n=7) corresponds to the time of solution exchange (Baburin et al., 2006). Such a slow deactivation usually accompanies enhanced desensitization (e.g. Jones and Westbrook, 1995; Haas and Macdonald, 1999). This was obviously not the case for I_{VA} (Fig. 23 A). The mechanism underlying slower kinetics of I_{VA} is currently not clear and warrants future studies. Direct activation of $GABA_A$ channels by VA may, however, also be explained by an interaction of VA with the GABA binding site as I_{VA} were efficiently inhibited by bicuculline (1 µM). However, it cannot be excluded that conformational changes induced by bicuculline indirectly affect VA action (see also Ueno et al., 1997).

These data show that VA is an agonist with much lower efficiency than GABA. The maximal current induced by the highest VA concentration of 300 µM (higher concentrations were not testable due to limited solubility) did not exceed 15% of the current induced by saturating (1 mM) GABA concentrations (Khom et al., 2007).

11.1.3 EVIDENCE FOR OPEN CHANNEL BLOCK

At high concentrations (≥30 µM) a “wash-off” current occurred upon washout of VA. A straightforward interpretation of this finding is that VA rapidly unbinds from its binding site within the open channel (see also Rho et al., 1996; Dalziel et al., 1999; Akk and Steinbach,

2000; Krampfl et al., 2002; Feng et al., 2004). Such a scenario is supported by the concentration-dependent increase of this current suggesting a concentration-dependent open channel block (Fig. 23). Further evidence for an open-channel block mechanism comes from the accelerated I_{GABA} decay in the presence of high concentrations of the structurally related acetoxy-VA (Fig 24 B). In order to further explore this possibility, the bell-shaped concentration-response curve that was typically seen for VA action was simulated. The corresponding IC_{50} (~190 µM) value that was estimated assuming that the accelerated I_{GABA} decay reflects open-channel block (Fig. 24 C) nicely agrees with the simulation of the bell-shaped concentration-response curve assuming a low affinity open-channel block mechanism for VA (simulated IC_{50} = 190 µM).

These findings suggest that VA and acetoxy-VA might act at the same site in the channel pore. The discussed open channel block mechanism remains however, hypothetical, because acetoxy-VA may also interact with a different binding site than VA.

Surprisingly, an enhanced I_{GABA} decay was observed only for acetoxy-VA and not for the structurally related hydroxy-VA. This finding suggests that substitution of the hydrogen in position 1 of the hexahydro-indene ring by a hydroxyl- or acetoxy-group defines not only the modulatory action but also the affinity for open $GABA_A$ channels.

The complex mechanism of VA action (including modulation of I_{GABA}, direct activation of $GABA_A$ channels and open channel block) complicates a straightforward estimation of the efficiencies and potencies of VA for the different subunit compositions. This is illustrated in Figs 19 B-D and 22 where the modulatory action is apparently superimposed by inhibitory action.

Taken together a subunit specific modulation of $GABA_A$ receptors by VA ($\beta_3 > \beta_2 >> \beta_1$-containing receptors, Fig. 22) was described. Positive allosteric modulation is caused by a VA-induced increase in the GABA sensitivity (Fig. 20). The threshold concentration (1 µM) of this modulatory action is in the range of the estimated plasma concentration of VA (Anderson et al., 2005). At high concentrations, VA activates $GABA_A$ channels directly (Fig. 23) and also blocks the channel. The clinical evidence for *Valerian* effects is, however, still controversial (see Sampson, 2005 and De Smet, 2002 for review). However, these data open the perspective that the proposed sedative, hypnotic and anxiolytic effects suggested for *Valerian* may be caused by interaction of VA with $GABA_A$ channels (Khom et al., 2007).

11.2 MODULATION OF $GABA_A$ RECEPTORS BY *ACTAEA RACEMOSA* EXTRACTS

Preparations made from the rhizome of *Actaea racemosa* are widely marked as herbal remedies for the treatment of menopausal symptoms. US sales figures in 2005 reached $9.7 million. The clinical efficacy of these extracts is still unclear (for review, see Borrelli and Ernst, 2008) and the precise mechanism of action is currently unknown. It was hypothesized that *Actaea racemosa* ingredients compete with estrogen for binding sites and exerts a positive estrogenic effect. Other data were interpreted that it may act as a selective estrogen receptor modulators, depending on the tissue receptors (Bodinet and Freudenstein, 2004), and that it may also exert an agonistic effect on serotonin receptors (Burdette et al., 2003). In addition, *Actaea racemosa* extracts may decrease luteinizing hormone levels, leading to a decrease in hot flashes (Borrelli and Ernst, 2002; Carroll, 2006). Vermes et al. (2005) reported a significant reduction of insomnia- and anxiety-related disorders in menopause by an isopropanol extract (Remifenin®) of *Actaea racemosa* containing the triterpene glycoside actein.

The modulation of I_{GABA} by 3 different Actaea racemosa extracts (kindly provided by Univ.Prof. DDr. Brigitte Kopp and colleagues Mag. Gabriele Trauner and Mag. Ulrike Jäger) was examined by means of the two-microelectrode voltage clamp techniqu (see Fig. 27). All the tested extracts exhibited a positive modulatory effect.

11.2.1 ACTEIN MODULATION OF $GABA_A$ RECEPTORS COMPOSED OF DIFFERENT α OR β SUBUNIT VARIANTS

Actein was identified as an efficient modulator of $GABA_A$ receptors (see Figs. 28 and 29).

The modulation of heterologously in *Xenopus* oocytes expressed $GABA_A$ receptors comprising different subunit combinations (see Table 3) by actein was studied, as illustrated in Figs. 28 and 29, half-maximal stimulation of I_{GABA} occurred at concentrations ≥30 μM. Maximal I_{GABA} enhancement occurred at 300 μM of actein (see Figures 28 A and 29 A, see also Table 3). At concentrations ≥30 μM actein directly activated $GABA_A$ receptors (i.e. in the absence of GABA). In order to obtain insight into the underlying molecular basis, the modulation of $GABA_A$ channels composed of different α, β and γ subunits was investigated.

As illustrated in Figs. 28 A and 29 A, actein modulated all tested receptor subtypes, irrespective of the subunit composition. However, different efficiencies were observed for different receptor subtypes (see Figs., 28 A and 29 B, Table 3).

Most pronounced modulation of I_{GABA} occurred at $\alpha_1\beta_1$ receptors, whereas only a weak stimulation was observed in $\alpha_5\beta_2$. The order of I_{GABA} stimulation was $\alpha_1\beta_1>\alpha_1\beta_3>\alpha_2\beta_2>\alpha_1\beta_2>\alpha_3\beta_2>\alpha_1\beta_2\gamma_{2S}>\alpha_6\beta_2\gamma_{2S}>\alpha_4\beta_2\gamma_{2S}>>\alpha_5\beta_2$ (see Figs., 28 A and 29 A, Table 3).

11.2.2 ACTEIN MODULATES I_{GABA} INDEPENDENT ON THE GABA CONCENTRATION

Actein (300 μM) modulated I_{GABA} induced by different GABA concentrations to a comparable extent. The GABA concentration-effect curve in the presence of 300 μM actein was shifted towards higher GABA sensitivity and $I_{max\text{-}GABA}$ was increased (see Fig. 30 A). At concentrations ≥30 μM actein directly gated $GABA_A$ receptors irrespective of the subunit composition, however the currents induced by actein did not exceed 2% of $I_{max\text{-}GABA}$. Similar pharmacological features have been previously reported for other allosteric modulators such as barbiturates (Schulz and Macdonald, 1981; Thompson et al. 1996; Feng et al. 2004), anaesthetics such as etomidate (Hong and Wang, 2005) or neurosteroids such as THDOC (Lambert et al., 2003; Hosie et al., 2006).

11.2.3 *IN VIVO* EFFECTS OF ACTEIN

Open field test

A decrease in locomotor activity in the OF test represents a reliable measure for sedative-like effects in murines (Cheng et al. 2006). Actein significantly decreased the distance travelled by mice in the OF test compared to their vehicle-treated littermates (see Fig. 31). At 6 mg/kg KG and 20 mg/kg KG actein, respectively, animals covered half of the distance compared to the control group suggesting a sedative effect. Interestingly, 0.6 mg/kg KG actein induced a slight increase in the distance travelled in the centre as well as in the time spent in the centre. This increase, which may indicate an anxiolytic action of actein, however, was not significant. Concentrations higher than 60 mg/kg did not further decrease the locomotor activity. However, this effect is presumably not due to reaching saturation, but more likely due to the poor solubility of actein in the vehicle. Previously, it has been suggested that α_1 containing receptors mainly contribute to the sedative effects of ligands of the benzodiazepine recognition site (Rudolph et al., 1999; Low et al., 2000; Crestani et al., 2001). This is in agreement with the results of the electrophysiological experiments in the *Xenopus* assay, showing a strong effect of actein on $GABA_A$ receptors containing α_1-subunits (see Fig. 28 A and 29 A, see also Table 3).

Elevated Plus Maze and Light- Dark Choice Test

A trend towards an anxiolytic effect of actein was observed in the OF test. In the EPM test, actein caused an increase of the time spent in the open arms including visits of open arms (see Fig. 30). This behaviour is commonly associated with an anxiolytic activity of the tested compound (Crawley, 2008). However, this effect was not significant and at higher concentrations (≥2 mg/kg) actein even reduced a significant decrease in the time spent in the open arms of the elevated-plus-maze (data not shown). Nonetheless, this apparent loss of anxiolytic-like activity was presumably due to a developing sedative-like effect. In the LDT test, actein significantly increased the time spent in the brightly lit arena, thus suggesting a retained anxiolytic effect (Fig. 31).

Stress-induced hyperthermia

Assessment of anxiolytic-like activity in the EPM and the LDT is highly dependent on an intact locomotor activity (Crawley, 2008). Actein revealed in the OF, EPM and LDT a trend towards anxiolytic activity, however the observed increases (see Figs. 30 and 31) were not significant compared to control (except in the LDT). In the SIH, 6 and 20 mg/kg actein caused a significant decrease of ΔT, supporting the idea that the anxiolytic effect is concurrently superimposed by the sedative activity. My data indicate that actein efficiently modulates $GABA_A$ channels composed of $\alpha_2\beta_2$ and $\alpha_3\beta_2$ subunits (see Fig. 228, Table 3). This is in line with data of Low et al. (2000), McKernan et al. (2000), Collins et al. (2002), Dias et al. (2005) and Atack et al. (2006) illustrating that $GABA_A$ receptors comprising either α_2 or α_3 apparently mediate the anxiolytic effects of ligands of the benzodiazepine binding site. Taken toghether these data support the idea of an anxiolytic potential of actein.

Home Cage Activity

In order to gain insight into the time of course of action, animals treated with actein were monitored for 24 hours. 3 hours after injection, the actein activity reached a maximum nearly completely abolishing any activity. After 12 hours neither control group nor actein-treated group showed any differences in their behaviour, suggesting that actein in contrast to the frequently prescribed benzodiazepines could induce less hang-over effect or daytime sedation (see for review e.g. Bateson, 2004). Future studies will have to be performed to test this hypothesis.

Taken together, actein was identified as a highly efficient modulator of $GABA_A$ receptors. *In-vivo,* actein was shown to induce sedation and concurrent anxiolysis. These data suggest that *Actaea racemosa* effects in menopause (Vermes et al., 2005; Cheema et al., 2008) may at least be partially explained by an allosteric modulation of $GABA_A$ receptors. Other mechanisms of action can, however, not be excluded.

12. SUMMARY

Valerian and *Actaea racemosa* extracts are commonly used as “herbal medicinal products”, while their mechanisms of action are largely unknown. In this study, I have demonstrated that *Valerian* and *Actaea racemosa* extracts at a concentration of 100 μg/ml enhance chloride currents through $GABA_A$ receptors (I_{GABA}).

Valerenic acid (VA) selectively enhances I_{GABA} through receptors incorporating β_2 or β_3 subunits and its effect is not γ-subunit dependent. The stimulatory effect of VA on $\alpha_1\beta_2$ receptors was substantially reduced by the point mutation β_2N265S (known to inhibit loreclezole action). VA displayed a significantly lower efficiency on channels incorporating α_4 subunits ($\alpha_4\beta_2\gamma_{2S}$ receptors). At high concentrations ($\geq$ 100 μM) VA and acetoxy-VA inhibit I_{GABA} suggesting an open-channel block. In summary, VA was identified as a subunit-specific allosteric modulator of $GABA_A$ receptors that is likely to interact with the loreclezole binding pocket.

Actein (a compound isolated from *Actaea racemosa)* was identified as an efficient modulator of $GABA_A$ receptors displaying the strongest effect on $\alpha_1\beta_1$ receptors (2169.4±374.5%), whereas on $\alpha_5\beta_2$ containing receptors a substantially weaker efficiency was observed (225.8±44.8%). Actein (6 mg/kg) significantly decreased the spontaneous motor activity of mice in the open field test. A significant decrease of ΔT (difference of basal temperature before and after actein injection; 6 and 20 mg/kg actein) was observed in the stress-induced hyperthermia test. These *in vivo* data suggest that sedative and anxiolytic properties of actein may contributes to the treatment of postmenopausal disorders with *Actaea racemosa* extracts.

13. ZUSAMMENFASSUNG

Extrakte von Baldrian (*Valeriana officinalis*) und Traubensilberkerze (*Actaea racemosa*) finden Anwendung als „Phytopharmaka“. Ihre Wirkmechanismen sind unbekannt. In der vorliegenden Arbeit konnte gezeigt werden, dass sowohl Baldrian- als auch Traubersilberkerzen-Extrakte eine deutliche Stimulation von Chloridströmen (I_{GABA}) durch GABA Rezeptoren induzierten.

Valerensäure (VA), ein wichtiger Inhaltsstoff des Baldrians, wurde als Untereinheiten-spezifischer Modulator von $GABA_A$ Rezeptoren identifiziert, da ausschließlich $\beta_{2/3}$ Untereinheiten inkorporierende Rezeptoren positiv moduliert wurden. Das Einführen der Punktmutation β_2N265S (welche die Modulation von $GABA_A$ Rezeptoren durch Loreclezol hemmt) reduzierte die Modulation der Rezeptoren durch VA. VA zeigte außerdem eine geringere Effizienz an $\alpha_4\beta_2\gamma_{2S}$ $GABA_A$ Kanälen. In höheren Konzentrationen ($\geq$100 μM) blockierten VA und Acetoxy-VA I_{GABA} über einen “open-channel-block” Mechanismus. Zusammengefasst wurde VA als Untereinheiten-spezifischer Modulator von $GABA_A$ Rezeptoren identifiziert, der möglicherweise mit der Loreclezol-Bindungsstelle interagiert.

Actein, ein Inhaltsstoff der Traubensilberkerze, wurde als effizienter Modulator von $GABA_A$ Rezeptoren identifiziert, wobei die stärkste Potenzierung von I_{GABA} an $\alpha_1\beta_1$ Rezeptoren beobachtet (2169.4±374.5%) wurde, während der Effekt an $\alpha_5\beta_2$ Rezeptoren deutlich schwächer ausgeprägt war (225.8±44.8%).

Actein (6 mg/kg) reduzierte signifikant die spontane Motoraktivität der Mäuse im Open Field Test. Im Light/Dark Choice Test, verbrachten Actein-behandelte Mäuse im Vergleich mit Kontrolltieren signfikant mehr Zeit im hellbeleuchten Teil der Box. Eine deutliche Reduktion von ΔT (Differenz der Körpertemperatur vor und nach Actein-Injektion; 6 and 20 mg/kg Actein) wurde im Stress-induced Hyperthermia Test beobachtet. Diese Daten legen nahe, dass Actein *in vivo* über eine Interaktion mit $GABA_A$ Rezeptoren sedative und anxiolytische Wirkungen induzieren kann und dass diese Effekte möglicherweise zur beobachteten Linderung postmenopausaler Beschwerden beitragen.

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Publikationen

Khom S, Baburin I, Timin EN, Hohaus A, Sieghart W and Hering S (2006) Pharmacological properties of GABAA receptors containing gamma1 subunits. *Mol Pharmacol* **69**:640-9.

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Kurzvorträge

„Pharmacological properties of $GABA_A$-receptors containing γ_1- subunits“ **APHAR- Tagung Wien Nov 2005**

„A novel method for medium-throughput screening of herbal sedatives and benzodiazepine-like ligands on $GABA_A$-receptors expressed in *Xenopus* oocytes“ **ÖphG- Tagung Innsbruck April 2005**

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„Modulation of $GABA_A$ Receptors by Compounds of Natural Origin“ **Universität Innsbruck, Mai 2007**

“HPLC-based Activity Profiling of Plant and Fungal Extracts for $GABA_A$ Receptor Ligands using a Functional Assay with *Xenopus* Oocytes” **55th Annual Meeting of the Society Medicinal Plant Research, Graz, September 2007.**

“$GABA_A$ receptors: target for multiple compounds of natural origin” **Universität Wien, Initiativkolleg, Dezember 2007.**

“$GABA_A$ Rezeptoren: Molekulares Substrat von Substanzen biogenen Ursprungs und Extrakten mit sedativen und anxiolytischen Eigenschaften“ **AKH (Prof. Zeitlhofer), Februar 2008.**

“Drugs for the CNS: Focus on $GABA_A$ receptors using a functional assay on *Xenopus* Oocytes**“ Universität Wien,** März 2008**.**

Poster

Khom S, Baburin I, Timin EN, Hohaus A, Sieghart W and Hering S (2006) Pharmacological properties of GABAA receptors containing gamma1 subunits. **Neuroscience 2006, Atlanta, USA.**

Khom S, Baburin I, Timin EN, Hohaus A, Trauner G, Kopp B and Hering S (2007) Valerenic acid potentiates and inhibits $GABA_A$ receptors: Molecular mechanism and subunit specificity **Neuroscience 2007, San Diego CA, USA.**

Auszeichnungen

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Sprachkenntnisse

Deutsch: Muttersprache
Englisch: fließend
Französisch: fließend
Italienisch: gut