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$GABA_A$ RECEPTORS IN THE BRAIN: EXCITING TARGETS FOR THE DEVELOPMENT OF CLINICALLY IMPORTANT DRUGS


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Werner Sieghart

GABAA RECEPTORS IN THE BRAIN: EXCITING TARGETS FOR THE DEVELOPMENT OF CLINICALLY IMPORTANT DRUGS

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First of all I would like to thank the Hungarian Academy of Sciences for electing me as an Honorary Member and I would especially like to thank Peter Somogyi and all those who supported him in proposing me for this election. I am very honored by this distinction and will continue to support Hungarian Science to the best of my abilities.

In this lecture I will explain why I entered $GABA_A$ receptor research, why I stayed in this field, will mention some of my major scientific contributions, and will provide you with an up-to-date overview of our current knowledge on the structure, pharmacology and function of $GABA_A$ receptors. At the end of my presentation I hope to have convinced you that $GABA_A$ receptors are really exciting targets for the development of clinically important drugs.

The first visualization of neurons was achieved by Camillo Golgi in 1873. He soaked brain tissue with silver chromate, looked through a microscope, and identified pyramidal shaped neurons with large and heavily branched dendritic trees and thin and weakly or unbranched axons. The dendrites collect information from thousands of other neurons that can come as excitation (depolarization), or inhibition (hyperpolarization). If the excitatory input overcomes the inhibitory input as well as a certain threshold of excitation at the axon initial segment, the neuron fires and propagates its excitation via an action potential to many other neurons. At the synapses, the contact sites to other neurons, action potentials elicit the release of a chemical substance, a neurotransmitter, which diffuses through the synaptic cleft that separates the synapse from the postsynaptic neuron. At the postsynaptic membrane the

neurotransmitter interacts with a receptor and by that elicits an opening of an ion channel. The opening of a sodium channel causes an excitation of the postsynaptic neuron, the opening of a chloride channel an inhibition. Again the neuron sums up all information transmitted via multiple synapses and fires if excitation is strong enough and above the threshold of excitation of the neuron. Information processing - in the form of making decisions on whether some information is sufficiently strong and important to be further propagated to the brain or within the brain - thus occurs at each individual neuron and involves multiple pieces of information via inputs from different other neurons.

My involvement in $GABA_A$ receptor research started in 1978, when I returned from my two years' postdoctoral research in the lab of Paul Greengard at the Department of Pharmacology, Yale University, Connecticut, USA, to the University Clinic for Psychiatry, University Vienna, Austria. Although I had been scientifically extremely successful during my time as a postdoc with Paul Greengard by publishing a total of 8 papers in scientific journals and by demonstrating that protein phosphorylation is involved in secretion[1,2] and that Ca^{2+} and cAMP regulate the phosphorylation of the same nerve-specific proteins[3], I felt that I could not continue protein phosphorylation research at the University Clinic for Psychiatry because I needed a topic of interest for psychiatrists. Investigating the "benzodiazepine receptors" seemed to be such a topic. Benzodiazepines, such as chlordiazepoxide or diazepam, had been introduced into therapeutic use in the 1960´s, and due to their

[1] Sieghart W, Theoharides ThC, Alper SL, Douglas WW, and Greengard P (1978) Calcium dependent protein phosphorylation during secretion by exocytosis in the mast cell. *Nature* 275, 329-331.

[2] Theoharides ThC, Sieghart W, Greengard P, and Douglas WW. (1980) Antiallergic drug cromolyn may inhibit histamine secretion by regulating phosporylation of a mast cell protein. *Science* 207, 80-82.

[3] Sieghart W, Forn J, and Greengard P (1979) Calcium and cyclic AMP regulate the phosphorylation of the same two membrane-associated proteins specific to nerve tissue. *Proc Natl Acad Sci, USA* 76, 2475-2479.

anxiolytic, anticonvulsant, muscle relaxant, and sedative hypnotic properties, soon belonged to the most heavily prescribed drugs in current therapeutic use. These receptors for sure were of interest for psychiatrists.

In 1975, Willi Haefely, the former director of research at the pharmaceutical company Hoffmann la Roche, which marketed the benzodiazepines, proposed that the action of benzodiazepines might be associated with $GABA_A$ (γ-aminobutyric acid type A) receptors. $GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the brain and can be inhibited by bicuculline, in contrast to $GABA_B$ receptors, that are G-protein coupled receptors with a different structure, function, and pharmacology.

In 1977, a high affinity benzodiazepine binding site was identified in brain membranes[4,5] which exhibited all properties one would expect from the benzodiazepine receptors. Several labs started immediately to investigate these novel binding sites and their properties, and this binding site was also investigated in the lab to which I returned[6]. I was well aware of these binding studies, but at the beginning was only marginally involved because I rather wanted to identify the "benzodiazepine receptor". I therefore was quite excited when Manfred Karobath, the head of the laboratory at that time, returned from a scientific meeting where he learned from Hanns Möhler, that [^{3}H]flunitrazepam, which can be used as a benzodiazepine receptor ligand in reversible binding studies, irreversibly binds to these receptors under irradiation with UV light[7]. I immediately started with the respective experiments and incubated brain

4 Möhler H, and Okada T (1977) Benzodiazepine receptor: demonstration in the central nervous system. *Science* 198, 849-851

5 Braestrup C, and Squires RF (1977) Specific benzodiazepine receptors in rat brain characterized by high-affinity [^{3}H]diazepam binding. *Proc Natl Acad Sci USA* 74, 3805-3809.

6 Karobath M, and Sperk G (1979) Stimulation of benzodiazepine receptor binding by gamma-aminobutyric acid. *Proc Natl Acad Sci USA* 76, 1004-1006.

7 Möhler H, Battersby MK, Richards JG (1980) Benzodiazepine receptor protein identified and visualized in brain tissue by a photoaffinity label. *Proc Natl Acad Sci USA* 77, 1666-1670.

membranes with [3H]flunitrazepam and irradiated the samples with UV light. For these experiments I used membranes from cerebellum and hippocampus, because there were first hints that benzodiazepine receptors in cerebellum and hippocampus might be different. I then solubilized the membranes with sodium dodecylsulfate (SDS) and subjected the proteins to SDS-polyacrylamide gel electrophoresis and fluorography. Using this technique, the many thousand proteins of the membranes are separated from each other according to their molecular mass, and only those proteins can be visualized, that had been irreversibly labeled by [3H]flunitrazepam. The very first experiments I did were already extremely exciting: in cerebellum a single protein with apparent molecular mass of 51 kDa was photolabled by [3H]flunitrazepam, whereas in hippocampus not only this 51kDa protein, but at least one more protein with apparent molecular mass 55 kDa was labeled[8] *(Fig 1).* All these proteins were associated with benzodiazepine receptors, because irreversible labeling by [3H] flunitrazepam could be blocked by diazepam. All these proteins were associated with a $GABA_A$ receptor, because their photolabeling with [3H]flunitrazepam could be enhanced by GABA and this enhancement could be blocked by bicuculline. Finally, I used the triazolopyridazine Cl 218872, one of the first compounds that exhibited a higher potency for inhibition of [3H]flunitrazepam binding in cerebellum than in hippocampus, and demonstrated that Cl 218872 completely inhibited photolabeling of the protein with 51 kDa in cerebellum as well as in hippocampus, but only weakly inhibited photolabeling of the other proteins in hippocampus. These results for the first time provided evidence for a molecular heterogeneity of $GABA_A$ receptors and confirmed the hypothesis, that benzodiazepine receptors are closely associated with $GABA_A$ receptors[8].

[8] Sieghart W, and Karobath M (1980) Molecular heterogeneity of benzodiazepine receptors. *Nature* 286, 285-287.

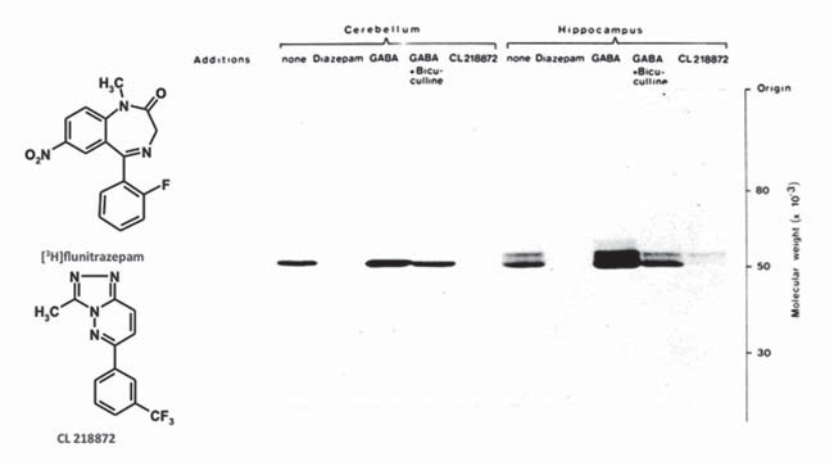

Figure 1: Structural formula of flunitrazepam and Cl 218872, and molecular heterogeneity of benzodiazepine receptors. Figure modified from Ref 8.

I was excited, the reviewers were excited, and the manuscript was rapidly published in "Nature". My excitement, however, soon disappeared when it became clear that some influential scientists in the field could not reproduce my data. They simply could not generate the high resolution SDS-gels I had learned to generate in the lab of Paul Greengard, and thus, could not separate the different photolabeled proteins in the hippocampus. I was desperate and asked all people who could not reproduce my data to come to my lab and to learn how to do that, and I promised to withdraw the paper if they then would not be able to reproduce the data in their own lab. Of course, nobody came to learn the technique, and I thus spent the next 8 years proving that I was right.

I improved the resolution of my gels, identified additional photolabeled proteins[9] and studied their regional distribution and postnatal development[10,11] which was distinct for each protein and different in different brain regions. In addition, I used several proteases and investigated the photolabeled fragments. The fragment pattern was different in cerebellum and hippocampus[12,13]. I did a much more extensive pharmacology of the photolabeling process just to find that the potency of the various benzodiazepine site ligands for the inhibition of irreversible labeling of the different proteins corresponded to that observed in reversible binding studies: ligands that exhibited the same affinity for benzodiazepine receptors in cerebellum and hippocampus exhibited the same potency for inhibition of irreversible [^{3}H]flunitrazepam binding to the different proteins, and ligands with higher affinity for receptors in cerebellum also exhibited a higher potency for inhibiting photolabeling of the 51 kDa protein, than of the proteins with higher molecular masses[14]. I treated the photolabeled proteins with various glycosidases to remove all sugar chains of possible different length. The molecular mass of all photolabled proteins was reduced by this treatment, but the difference in the apparent mass of proteins in the hippocampus was still there[15].

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9 Sieghart W, and Drexler G (1983) Irreversible binding of [^{3}H]flunitrazepam to different proteins in various brain regions. *J Neurochem* 41, 47-55.

10 Sieghart W, and Mayer A (1982) Postnatal development of proteins irreversibly labeled by [^{3}H] flunitrazepam. *Neurosci Letters* 31, 71-74.

11 Eichinger A, and Sieghart W (1986) Postnatal development of proteins associated with different benzodiazepine receptors. *J Neurochem* 46, 173-180.

12 Eichinger A, and Sieghart W (1985) Differential degradation of different benzodiazepine binding proteins by incubation of membranes from cerebellum or hippocampus with trypsin. *J Neurochem* 45, 219-226.

13 Sieghart W, Eichinger A, and Zezula J (1987) Comparison of tryptic peptides of benzodiazepine binding proteins photolabeled with [^{3}H]flunitrazepam or [^{3}H]Ro15-4513. *J Neurochem* 48, 1109-1114.

14 Sieghart W, Mayer A, and Drexler G (1983) Properties of [^{3}H]flunitrazepam binding to different benzodiazepine binding proteins. *Eur J Pharmacol* 88, 291-299.

15 Sieghart W, and Fuchs K (1988) Modification of the apparent molecular weight of different benzodiazepine binding proteins from rat brain membranes by various endoglycosidases. *Neurosci Letters* 86, 213-218.

In the meantime, two other groups were able to reproduce my data[16,17] and I became more relaxed. Nevertheless, I was relieved when the groups of Peter Seeburg and Eric Barnard published the first cloning of $GABA_A$ receptors[18]. Following the first purification of $GABA_A$ receptors from bovine brain by Erwin Sigel[19], two homologous subunits (α, β) were cloned, their putative transmembrane structure was determined (large N-terminal extracellular domain, four transmembrane domains (TMs), large intracellular loop between TM3 and TM4, short extracellular C-terminal domain) and it was demonstrated that only a combination of these subunits could produce a GABA activated chloride channel. One year later, the group of Peter Seeburg published the existence of several different homologous α subunits (α1, α2, α3) and the combination of these α subunits with the β subunit generated distinct receptors[20]. So 8 years after our publication in Nature, the heterogeneity of $GABA_A$ receptors had finally been confirmed. Again, one year later, the group of Peter Seeburg demonstrated that besides α and β subunits, a third subunit, a γ subunit, is required to generate $GABA_A$ receptors that also could be modulated by benzodiazepines[21]. These data thus finally confirmed the conclusion that benzodiazepine receptors are not only closely associated with

[16] Lippa AS, Garrett KM, Tabakoff B, Beer B, Wennogle LP, Meyerson LR (1985) Heterogeneity of brain benzodiazepine receptors: effects of physiological conditions. *Brain Res Bull* 14, 189-195.

[17] Hebebrand J, Friedl W, Unverzagt B, Propping P (1986) Benzodiazepine receptor subunits in avian brain. *J Neurochem* 47, 790-793.

[18] Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA et al. (1987) Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. *Nature* 328, 221-227.

[19] Sigel E, Mamalaki C, Barnard EA (1982) Isolation of a GABA receptor from bovine brain using a benzodiazepine affinity column. *FEBS Lett* 147, 45-48.

[20] Levitan ES, Schofield PR, Burt DR, Rhee LM, Wisden W, Köhler M, Fujita N, Rodriguez HF, Stephenson A, Darlison MG, et al., (1988) Structural and functional basis for $GABA_A$ receptor heterogeneity. *Nature* 335, 76-79.

[21] Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, Seeburg PH (1989) Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature* 338, 1491-1497.

$GABA_A$ receptors, but represent allosteric binding sites at at least some of these receptors.

A total of 6α, 3β, 3γ, δ, ε, π, θ, and 3 ρ subunits, as well as several alternatively spliced isoforms of some of these subunits were then cloned from the mammalian brain in the subsequent years[22]. Within a group (α, β, γ, δ, ε, π, θ, ρ) the subunits exhibited a homology of about 70%, in between the groups, the homology was about 30%. The homology of $GABA_A$ receptor subunits to subunits of other members of ligand gated ion channels (nACh receptors, glycine receptors, 5-HT3 receptors) was about 20%.

The amino acid sequences of the cloned $GABA_A$ receptor subunits allowed to generate receptor subtype-selective antibodies by using subtype-specific amino acid sequences for immunizing of rabbits. These first subunit-specific antibodies were used to demonstrate that the various proteins photolabeled by [^{3}H]flunitrazepam were various α subunit types[23]. After Richard Olsen had demonstrated that [^{3}H]muscimol can be used as a photoaffinity label for the GABA site of $GABA_A$ receptors, we also used this new affinity label and with the help of subunit-specific antibodies demonstrated that the different proteins photolabeled by [^{3}H]muscimol were various β-subunits of $GABA_A$ receptors[24]. At that time, Hanns Möhler asked me to investigate a new photolabel of $GABA_A$ receptors (the imidazobenzodiazepine Ro 15-4513) that seemed to have peculiar properties. When we investigated this photolabel in cerebellum and hippocampus, it became clear that [^{3}H]Ro 15-4513 was able to irreversibly label not only the 51 kDa protein in cerebellum, similar to [^{3}H]flunitrazepam, but

[22] Simon J, Wakimoto H, Fujita N, Lalande M, Barnard EA (2004) Analysis of the set of $GABA_A$ receptor genes in the human genome. *J Biol Chem* 279, 41422-41435.

[23] Fuchs K, Möhler H, and Sieghart W (1988) Various proteins from rat brain, specifically and irreversibly labeled by [^{3}H]flunitrazepam, are distinct alpha subunits of the GABA-benzodiazepine receptor complex. *Neurosci Letters 90, 314-319.*

[24] Fuchs K, and Sieghart W (1989) Evidence for the existence of several different α- and β-subunits of the GABA-benzodiazepine receptor complex from rat brain. *Neurosci Letters 97, 329-333.*

also an additional protein with apparent molecular mass 56 kDA[25,26]. Several years later, the α6 subunit of $GABA_A$ receptors was cloned and [^{3}H]Ro 15-4513 was demonstrated to bind to receptors containing this subunit[27]. Using our subsequently generated α6-selective antibodies, we demonstrated that this 56 kDa protein represents the α6 subunit of $GABA_A$ receptors that is located more or less exclusively in cerebellar granule cells.

To further investigate the heterogeneity of $GABA_A$ receptors, and to determine their subunit composition in vivo, my group generated large amounts of subunit-selective antibodies for synthesizing immunoaffinity columns. These columns allowed to enrich $GABA_A$ receptors containing the respective subunit and to determine their subunit composition. We for the first time demonstrated that the "type 1 benzodiazepine receptors" that are especially enriched in the cerebellum represent receptors containing the α1, a β and a γ2 subunit, and that the "type 2 benzodiazepine receptors" enriched in the hippocampus, consisted of α2 or α3 subunits together with a β and a γ2 subunit[28]. Whenever we generated antibodies against a novel subunit, these antibodies were used to purify receptors containing these subunits and to

[25] Möhler H, Sieghart W, Richards JG, and Hunkeler W (1984) Photoaffinity labeling of benzodiazepine receptors with a partial inverse agonist. *Eur J Pharmacol.* 102, 191-192.

[26] Sieghart W, Eichinger A, Richards JG, and Möhler H (1987) Photoaffinity labeling of benzodiazepine receptor proteins with the partial inverse agonist [^{3}H]Ro15-4513; a biochemical and autoradiographic study. *J Neurochem* 48, 46-52.

[27] Lüddens H, Pritchett DB, Köhler M, Killisch I, Keinänen K, Monyer H, Sprengel R, Seeburg PH (1990) Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. *Nature* 346, 648-651.

[28] Zezula J, and Sieghart W (1991) Isolation of type I and type II $GABA_A$-benzodiazepine receptors by immunoaffinity chromatography. *FEBS-Letters* 284, 15-18.

determine their subunit composition[29,30,31,32,33]. It soon became clear that there is an enormous heterogeneity of $GABA_A$ receptors in the brain: antibodies specific for a single α or β subunit type were able to co-purify most if not all other subunits, suggesting that there must be at least 2 α and 2 β subunits in $GABA_A$ receptors. In contrast, antibodies specific for a γ subunit co-purified all subunits except δ subunits, and antibodies against δ-subunits purified all subunits except γ subunits[31, 32 , 34, 35]. So γ and δ subunits seem not to occur together in the same receptor type and thus, seem to be present only once within $GABA_A$ receptors. All these results were confirmed by other groups. Combined with the finding of the group of Eric Barnard, that $GABA_A$ receptors are composed of 5 subunits that form a central chloride ion channel[36], it is now generally accepted that most of the $GABA_A$ receptors are composed of 2α, 2β, and one γ or δ subunit. My group was the first that not only determined the subunit composition, but also the subunit arrangement within

[29] Zezula J, Fuchs K, and Sieghart W (1991) Separation of α1-, α2- and α3-subunits of the $GABA_A$-benzodiazepine receptor complex by immunoaffinity chromatography. *Brain Res* 563, 325-328.

[30] Kern W, and Sieghart W (1994) Polyclonal antibodies directed against an epitope specific for the α4-subunit of $GABA_A$ receptors identify a 67 kDa protein in rat brain membranes. *J Neurochem* 62, 764-769.

[31] Tögel M, Mossier B, Fuchs K, and Sieghart W (1994) $GABA_A$ receptors displaying association of γ3-subunits with β2/3- and different α-subunits exhibit unique pharmacological properties. *J Biol Chem* 269, 12993-12998.

[32] Mossier B, Tögel M, Fuchs K, and Sieghart W (1994) Immunoaffinity purification of γ-aminobutyric $acid_A$ ($GABA_A$) receptors containing γ1-subunits. Evidence for the presence of a single type of γ-subunit in $GABA_A$ receptors. *J Biol Chem* 269, 25777-25782.

[33] Bencsits E, Ebert V, Tretter V, and Sieghart W (1999) A significant part of native γ-aminobutyric $acid_A$ receptors containing α4 subunits do not contain γ or δ subunits. *J Biol Chem* 274, 19613-19616.

[34] Jechlinger M, Pelz R, Tretter V, Klausberger T, and Sieghart W (1998) Subunit composition and quantitative importance of heterooligomeric receptors: $GABA_A$ receptors containing α6 subunits. *J Neurosci* 18, 2449-2457.

[35] Pöltl A, Hauer B, Fuchs K, Tretter V, Sieghart W (2003) Subunit composition and quantitative importance of $GABA_A$ receptor subtypes in the cerebellum of mouse and rat. *J Neurochem* 87, 1444-1455.

[36] Nayeem N, Green TP, Martin IL, Barnard EA (1994) Quaternary structure of the native $GABA_A$ receptor determined by electron microscopic image analysis. *J Neurochem* 62, 815-818.

$GABA_A$ receptors: these pentameric receptors are composed of alternating α and β subunits connected by a γ subunit[37]. The subunit composition and arrangement of receptors containing ε, θ, or π subunits currently is not clear, but ρ subunits seem to be able to form homo-oligomeric receptors, or hetero-oligomeric receptors with other ρ subunits.

Our antibodies, of course, were also used for immunohistochemical studies. The first study was performed together with the group of Hans Lassmann in Vienna, and demonstrated a differential regional distribution of α1, α2, and α3 subunits in the rat brain, further supporting the heterogeneity of $GABA_A$ receptors[38]. Peter Somogyi became alerted by this study and asked whether he could obtain the antibodies. I provided the antibodies and soon was very excited when I learned that especially the antibody against the α1 subunit was excellent and suitable for electron microscopy. This was the starting point of a long and prosperous collaboration with Peter Somogyi and Zoltan Nusser, and of a series of landmark papers published together. Using electron microscopic techniques these papers for the first time demonstrated a synaptic co-localization of α1 and β2/3 and γ2 subunits[39,40], that the α6 subunit of $GABA_A$ receptors is concentrated in both inhibitory and excitatory synapses on cerebellar granule cells[41], that different $GABA_A$ receptor subunits can exhibit

[37] Tretter V, Ehya N, Fuchs K, and Sieghart W (1997) Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. *J Neurosci* 17, 2728-2737.

[38] Zimprich F, Zezula J, Sieghart W, and Lassmann H (1991) Immunohistochemical localization of the α1-, α2- and α3-subunit of the $GABA_A$ receptor in the rat brain. Neurosci *Letters* 127, 125-128.

[39] Nusser Z, Roberts DBJ, Baude A, Richards JG, Sieghart W, and Somogyi P (1995) Immunocytochemical localization of the α1 and β2/3 subunits of the $GABA_A$ receptor in relation to specific GABAergic synapses in the dentate gyrus. *Eur J Neurosci* 7, 630-646.

[40] Somogyi P, Fritschy JM, Benke D, Roberts JDB, and Sieghart W (1996) The γ2 subunit of the $GABA_A$ receptor is concentrated in synaptic junctions containing the α1 and β2/3 subunits in hippocampus, cerebellum and globus pallidus. *Neuropharmacol* 35, 1425-1444.

[41] Nusser Z, Sieghart W, Stephenson FA, and Somogyi P (1996) The α6 subunit of the $GABA_A$ receptor is concentrated in both inhibitory and excitatory synapses on cerebellar granule cells. *J Neurosci* 16, 103-114.

a differential subcellular distribution on hippocampal pyramidal neurons[42], and that in addition to synaptic receptors there are also extrasynaptic receptors in cerebellar granule cells[43]. Whereas γ2-containing receptors could be found at synapses as well as extrasynaptically, δ-containing receptors seem to be located exclusively extrasynaptically.

Other researchers asked for our antibodies too, and this resulted in a series of collaborative studies on the localization of various $GABA_A$ receptor subunits in different brain regions and cell types, their changes in development and under the influence of ethanol. Such studies also provided important information on the co-localization of $GABA_A$ receptor and glycine receptor subunits at synapses in the spinal cord[44] and on subunit partnerships within $GABA_A$ receptors, indicating that α6 and δ subunits are strongly associated with each other[45]. Other collaborations indicated that the neurosteroid sensitivity of δ subunit knockout mice is significantly decreased, suggesting that neurosteroids predominantly act via δ-containing receptors[46].

In 1996 I was asked by Günther Sperk whether he could obtain our antibodies for investigating the regional distribution of $GABA_A$ receptor

[42] Nusser Z, Sieghart W, Benke D, Fritschy JM, and Somogyi P (1996) Differential synaptic localization of two major γ-aminobutyric acid type A receptor α subunits on hippocampal pyramidal cells. *Proc Natl Acad Sci USA* 93, 11939-11944.

[43] Nusser Z, Sieghart W, and Somogyi P (1998) Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J Neurosci* 18, 1693-1703.

[44] Todd AJ, Watt C, Spike RC, and Sieghart W (1996) Co-localization of GABA, glycine and their receptors at synapses in the rat spinal cord. *J Neurosci* 16, 974-982.

[45] Jones A, Korpi ER, McKernan RM, Nusser Z, Pelz R, Mäkelä R, Mellor JR, Pollard S, Bahn S, Stephenson RA, Randall AD, Sieghart W, Somogyi P, Smith AJH, and Wisden W (1997) Ligand-gated ion channel subunit partnerships: $GABA_A$ receptor α6 subunit gene inactivation inhibits δ subunit expression. *J Neurosci 17, 1350-1362*.

[46] Mihalek RM, Banerjee PK, Korpi ER, Quinlan JJ, Firestone LL, Mi -PZ, Lagenaur C, Tretter V, Sieghart W, Anagnostaras SG, Sage JR, Fanselow MS, Guidotti A, Spigelman I, Li Z, DeLorey TM, Olsen RW, and Homanics GE (1999) Attenuated sensitivity to neuroactive steroids in γ-aminobutyrate type A receptor delta subunit knockout mice. *Proc Natl Acad Sci USA* 96, 12905-12910.

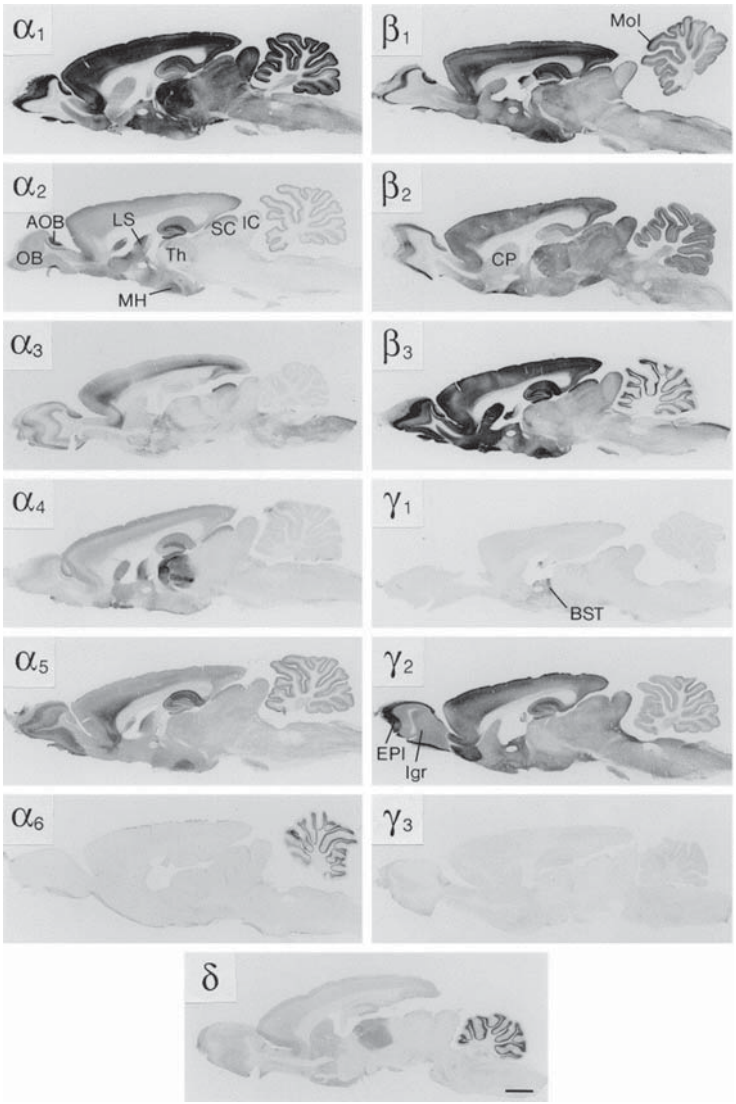

Figure 2: Regional distribution of 13 $GABA_A$ receptor subunits in the rat brain. Figure taken from Ref 49.

subunits in the rat brain. Since Peter Somogyi was not interested in a systematic investigation of the regional distribution of subunits, I started the collaboration with Günther Sperk. From several hundred subunit-specific antibodies sent to him he selected the best ones for immunohistochemistry of 13 different subunits. We first published the differential distribution of the 13 subunits in hippocampus and the changes in this distribution occurring during epileptic seizures[47,48]. Later on we published the distribution of these 13 subunits in the whole rat brain[49] *(Fig 2)* and recently in the whole mouse brain[50].

In 2001 the crystal structure of the acetylcholine binding protein (AChBP) was published by the groups of Titia Sixma and Guus Smit[51]. This protein forms homopentamers, and is secreted into the synaptic cleft of a snail where it binds acetylcholine. Due to its structural homology to the extracellular domain of nicotinic acetylcholine receptors this protein is also homologous to the extracellular domain of $GABA_A$ receptors and could thus be used as a template for modelling this extracellular receptor domain. Margot Ernst in my lab, supported by an undergraduate student and Stefan Boresch from the Institute of Theoretical Chemistry and Structural Biology in Vienna, was one of the first to model the extracellular domain of $GABA_A$ receptors by using the

[47] Sperk G, Schwarzer C, Tsunashima K, Fuchs K, and Sieghart W (1997) $GABA_A$ receptor subunits in the rat hippocampus I: Immunocytochemical distribution of thirteen subunits. *Neurosci* 80, 987-1000.

[48] Schwarzer C, Tsunashima K, Wanzenböck C, Fuchs K, Sieghart W, and Sperk G (1997) $GABA_A$ receptor subunits in the rat hippocampus II: Altered distribution in kainic acid-induced temporal lobe epilepsy. *Neurosci* 80, 1001-1017.

[49] Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neurosci* 101, 815-850.

[50] Hörtnagl, H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G (2013) Patterns of mRNA and protein expression for 12 $GABA_A$ receptor subunits in the mouse brain. *Neurosci* 236, 345-372

[51] Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van Der Oost J, Smit AB, Sixma TK (2001) Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* 411, 269-276.

crystal structure of the AChBP as a template[52]. She also carefully analyzed the homologies of the different parts of the AChBP and $GABA_A$ receptor structure and provided some guidelines on the reliabilities of the different parts of the model structure. Together with mutagenesis studies from a variety of authors and studies using concatenated subunits of Erwin Sigel, these data for the first time identified the absolute arrangement of subunits within $GABA_A$ receptors and the location of the two GABA binding sites at the two β+α– interfaces and that of the benzodiazepine binding site at the α+γ– interface *(Fig3A)*.

At that time it was already clear that $GABA_A$ receptors are chloride ion channels that can be opened by GABA and can be allosterically modulated by a large variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, neuroactive steroids, anesthetics, convulsants and many other compounds[53]. All these compounds seem to act via distinct binding sites at $GABA_A$ receptors. Whereas benzodiazepines can only modulate ongoing GABAergic activity and cannot directly activate $GABA_A$ receptors in the absence of GABA, most of these other compounds at low concentrations allosterically modulate GABA-induced ion flux, but at higher concentrations are also able to directly activate these receptors in the absence of GABA. These compounds thus exhibit a much higher toxicity than benzodiazepines. In addition, depending on the structure of the benzodiazepines, their binding to the benzodiazepine binding site can induce or stabilize different conformations of the receptor that can either enhance or reduce GABA-induced ion flux. A third group of ligands bind to the benzodiazepine site but do not change GABA-induced currents. These compounds, however, can block the effect of other ligands acting via the benzodiazepine binding site[53]. Given the rich and

[52] Ernst M, Brauchart D, Boresch S, and Sieghart W (2003) Comparative modeling of $GABA_A$ receptors: limits, insights, future developments. *Neurosci* 119, 933-943.

[53] Sieghart W. (1995) Structure and pharmacology of $GABA_A$ receptor subtypes. *Pharmacol Rev* 47, 181-234.

complex pharmacology of $GABA_A$ receptors, I always wondered how a single receptor could accomodate so many binding sites, and where these binding sites might all be located.

The answer again came from modelling studies. In 2005, Nigel Unwin published the structure of not only the extracellular domain but also of the transmembrane domains of the nACh receptor from cryo-electronmicroscopic studies. This structure was then used as a template for modelling the $GABA_A$ receptor extracellular and transmembrane domains by Margot Ernst[54] *(Fig3C).* In doing that she soon realized that there are quite a few solvent accessible spaces within the $GABA_A$ receptor. In the extracellular domain of the pentameric receptor, the five subunit interfaces are obviously solvent accessible. Each subunit exhibits four transmembrane helices and at the interface between two such four-helix bundles, between helix 1 of the one subunit and helix 3 of the neighbouring subunit, another solvent accessible space is located. A third type of solvent accessible spaces is located within each four helix bundle, and of course, the ion channel itself is solvent accessible *(Fig3B).* This total of 16 solvent accessible spaces (pockets) presumably are used for conformational changes of the receptors, but on binding of compounds within these pockets, the compounds might induce or stabilize a certain confomational state of the receptor that enhances or reduces GABA-induced ion flux. In addition, it is conceivable that the synergistic effect of two ligands binding in different pockets could also directly activate the receptor in the absence of GABA. These data were consistent with mutagenesis studies identifying amino acid residues important for the binding or transduction of the effects of inhalation- or intravenous-anesthetics and of steroids. Recently, the concept of the existence of binding sites for steroids and anesthetics within the transmembrane domains

[54] Ernst M, Bruckner S, Boresch S, and Sieghart W. (2005) Comparative models of $GABA_A$ receptor extracellular and transmembrane domains: important insights in pharmacology and function. *Mol Pharmacol* 68, 1291-1300.

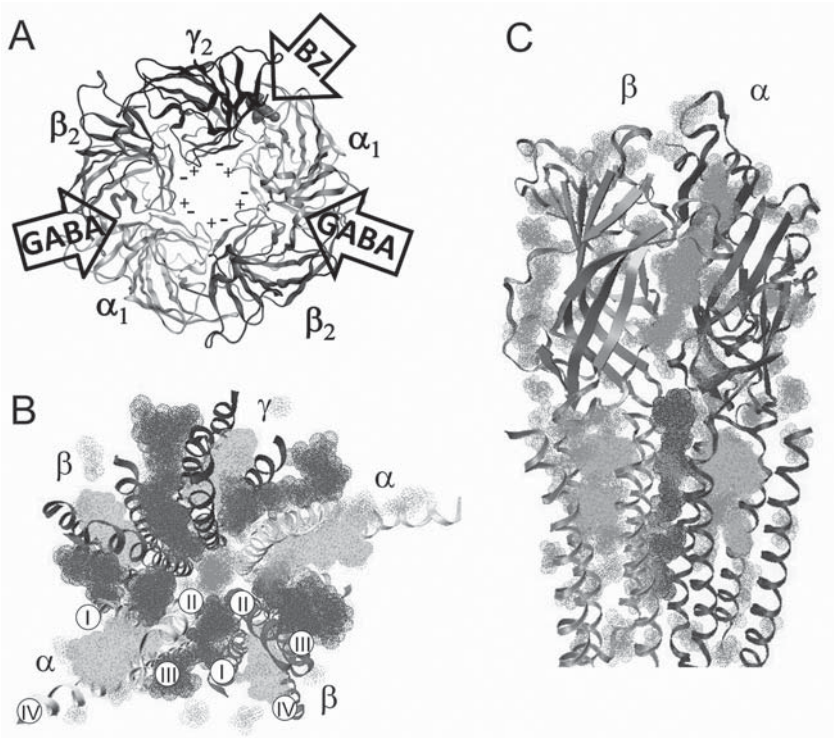

Figure 3: Model structure of the $GABA_A$ receptor. (A) Model of the extracellular domain of the $GABA_A$ receptor, giving the absolute arrangement for α1, β2, and γ2 containing receptors, the view is from extracellular. The + (plus) and - (minus) sides of the subunits are identified on the inner circumference of the channel. The location of the two GABA binding sites at the β2+α1- interfaces and the benzodiazepine (BZ) binding site at the α1+γ2- interface is indicated by arrows. The figure is a modification of the figure in Ref 52. (B) Solvent accessible spaces contained in $GABA_A$ receptor transmembrane models. The view is from outside the cell with the extracellular domain invisible. The four transmembrane helical domains of each subunit are shown. Solvent accessible spaces are within each transmembrane four helical domaine (pale gray) as well as between helix 1 of one subunit and helix 3 of the neighboring subunit (dark gray), as well as within the central ion channel (intermediate gray). (C) Dimer of the extracellular and transmembrane domaine of β and α subunits (GABA binding site) viewed from the outside of the pore. Solvent accessible spaces are within the extracellular interface (intermediate gray), within the continuation of this space in the transmembrane domaine between two four helix bundles (dark gray), within the four helix bundle of each subunit (pale gray). Figures (B) and (C) are modifications of figures shown in Ref. 54.

was confirmed by us and other groups using etomidate, propofol, or steroid derivatives that could be used for covalent labeling of amino acid residues located within the respective binding sites[55,56,57,58].

We, however, were especially interested in the benzodiazepine binding site, because benzodiazepines had been used clinically for more than 50 years already and there was still no solid information on how these drugs bind into the benzodiazepine binding site of $GABA_A$ receptors. In the absence of a $GABA_A$ receptor crystal structure, we could only use structural models for the docking of benzodiazepines into the benzodiazepine binding site. At the time this work was started, however, multiple crystal structures of proteins were available that are homologous to the extracellular domain of the $GABA_A$ receptor. These structures were obtained from different snails (AChBP from *lymnea stagnalis*, *aplysia californica*), from the nicotinic acetylcholine receptor, from the bacteria *Gloeobacter violaceus* (GLIC) or *Erwinia chrysanthemi* (ELIC), or from the nematode *Caenorhabditis elegans* (GluCl), often co-crystallized with different ligands. Each one of these structures could be used as a template for modelling $GABA_A$ receptors. These proteins, however, differed in their homology to $GABA_A$ receptors and depending on the ligand bound, had different conformations. A single model thus could not cover the variability of all templates.

[55] Li GD, Chiara DC, Sawyer GW, Husain SS, Olsen RW, Cohen JB (2006) Identification of a $GABA_A$ receptor anesthetic binding site at subunit interfaces by photolabeling with an etomidate analogue. *J Neurosci* 26, 11599-11605.

[56] Chiara DC, Dostalova Z, Jayakar SS, Zhou X, Miller KW, Cohen JB (2012) Mapping general anesthetic binding site(s) in human α1β3 γ-aminobutyric acid type A receptors with [^{3}H] TDBzl-etomidate, a photoreactive etomidate analogue. *Biochemistry* 51, 836-847.

[57] Chen ZW, Manion B, Townsend RR, Reichert DE, Covey DF, Steinbach JH, Sieghart W, Fuchs K, Evers AS (2012) Neurosteroid analogue photolabeling of a site in the TM3 domain of the β3 subunit of the $GABA_A$ receptor. *Mol Pharmacol* 82, 408-419.

[58] Yip GM, Chen ZW, Edge CJ, Smith EH, Dickinson R, Hohenester E, Townsend RR, Fuchs K, Sieghart W, Evers AS, Franks NP (2013) A propofol binding site on mammalian $GABA_A$ receptors identified by photolabeling. *Nature Chem Biol* 9, 715-720.

Margot Ernst, supported by Lars Richter, a gifted graduate student, therefore started to develop a novel docking procedure. Starting from 8 different crystal structures as templates and using multiple sequence alignments they ended up with 37 structural models of the extracellular domain of $GABA_A$ receptors. They then used the structure of the benzodiazepine diazepam for the selection of models suitable for docking: the two lipophilic regions of diazepam (the pendant phenyl ring and the annealed phenyl ring) had to be in lipophilic parts of the benzodiazepine pocket and diazepam had to have contacts with both subunits. Models not consistent with these requirements were eliminated. In a second step, 9 diazepam analogues with different substitutions at the ring system but with high affinity for the benzodiazepine binding site were used in a similar way to further reduce the number of docking-suitable models to a total of six. The docking of 9 diazepam analogues into 6 models, using flexible side chains and a lipophilic interaction above median resulted in a total of 1463 ligand poses in the benzodiazepine pocket that still could exhibit all orientations. After dividing these poses into 30 pose-clusters, only 3 clusters were identified that accomodated all 9 ligands, thus resulting in 3 common binding modes. Only one of these was supported by all experimental results available from more than 20 years of research. After the identification of the amino acid residues of the pocket in contact with the ligands, the first model structures of the ligand-bound benzodiazepine binding site were obtained[59] *(Fig 4).*

These structures are no crystal structures but represent a cluster of poses from different models. Nevertheless, they can be used like crystal structures. From a correct structure one would expect that it should also be able to bind additional ligands of the benzodiazepine binding site representing different

[59] Richter L, de Graaf C, Sieghart W, Varagic Z, Mörzinger M, de Esch IJP, Ecker GF, Ernst M (2012) Diazepam-bound $GABA_A$ receptor models identify new benzodiazepine binding site ligands. *Nature Chem Biol* 8, 455-464.

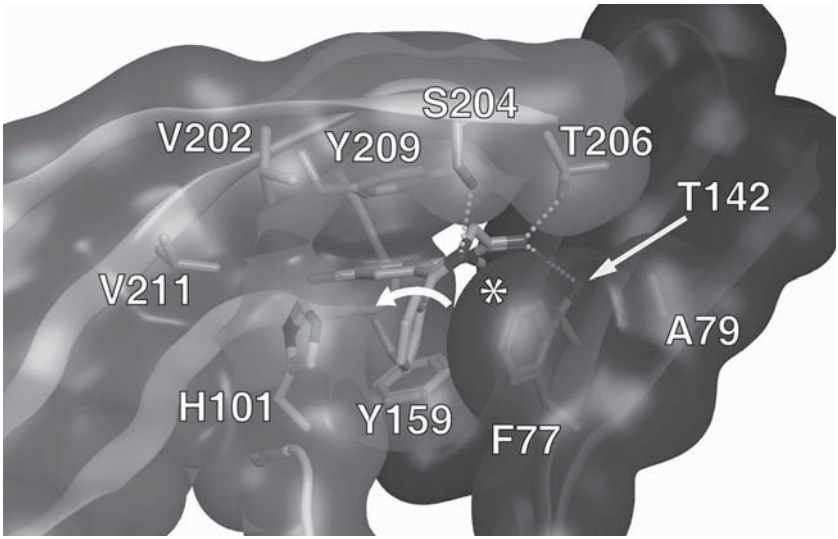

Figure 4: Representation of a diazepam docking pose within the benzodiazepine binding site, taken from Ref. 59.

structural classes. Retrospective virtual drug screening with a large database of 93.597 drug structures that was spiked with 41 benzodiazepine binding site ligands from different structural classes resulted in 8 benzodiazepine site ligands in the top 1% and an enrichment factor over random of 17.8. This enrichment factor was comparable to enrichment factors usually obtained with similar procedures from crystal structures. Further experiments will have to be performed to identify binding poses and conformations of the benzodiazepine pocket that are able to accomodate other ligands of the benzodiazepine binding site. But we also would have had to do that if we had started our docking from a crystal structure of a $GABA_A$ receptor. And such a structure is still not available. Nevertheless, this model structure is suitable for structure and fragment based drug design. This is indicated by a prospective docking based

virtual screening using the same library, as well as by a fragment based virtual screening using another compound library, that identified 3 structurally novel ligands of the benzodiazepine binding site that acted as positive allosteric modulators of $GABA_A$ receptors[59].

Given the existence of at least 41 distinct structural classes of benzodiazepine binding site ligands it is currently not very rewarding to identify structurally novel ligands for this site, although such novel ligands might be more suitable than existing ones for specifically modulating $GABA_A$ receptor subtypes. Structural models of $GABA_A$ receptor subtypes, however, would be interesting targets for structure- and fragment-based drug design and could be used for a rapid development of drugs with an interesting preclinical and possibly also clinical action. The classical benzodiazepines such as diazepam exhibit a more or less similar positive allosteric modulation at all $GABA_A$ receptors composed of $\alpha 1\beta\gamma 2$, $\alpha 2\beta\gamma 2$, $\alpha 3\beta\gamma 2$, or $\alpha 5\beta\gamma 2$ subunits, and by that, mediate their anxiolytic, anticonvulsant, muscle relaxant, and sedative-hypnotic actions. In the last 10 years the first benzodiazepine site ligands have been developed that could distinguish between these different $GABA_A$ receptor subtypes. In addition, investigating the loss of in vivo diazepam action in $GABA_A$ receptor subunit knockout mice, or in mice in which a point mutation in the (+) side of various α subunits was introduced, which renders the respective receptors diazepam-insensitive, it is now clear that $\alpha 1\beta\gamma 2$ receptors seem to mediate the sedative, anticonvulsant and anterograde amnestic properties of diazepam[60,61].

[60] Rudolph U, Crestani F, Benke D, Brünig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Möhler H (1999) Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. *Nature* 401, 796-800.

[61] McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P, Garrett L, Bristow L, Marshall G, Macaulay A, Brown N, Howell O, Moore KW, Carling RW, Street LJ, Castro JL, Ragan CI, Dawson GR, Whiting PJ (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the $GABA_A$ receptor alpha1 subunit. *Nat Neurosci* 3, 587-592.

Receptors composed of α2βγ2 predominantly mediate anxiolytic, muscle relaxant, and analgetic activity[62], as do α3βγ2 receptors. The latter receptors, however, require higher receptor occupancy for their anxiolytic action[63]. The analgetic action of α2βγ2 and α3βγ2 receptors is based on the fact that these receptor types are located on intrinsic dorsal horn neurons whereas α2βγ2 receptors are also expressed by primary afferents of the spinal cord[64]. Interestingly, there seems to be no tolerance development to these effects and thus, selectively enhancing the action of GABA at these receptor subtypes might find a highly interesting clinical application. In addition, modulating α2βγ2 and/or α3βγ2 receptors might have beneficial effects for the treatment of Down syndrome, affective disorders, schizophrenia and autism[65].

Receptors composed of α5βγ2 subunits again contribute to the myorelaxant action of diazepam but also are involved in regulating learning and memory. Mice, in which the α5 subunit has been deleted exhibit better cognitive abilities and learn better[65]. Negative allosteric modulators selective for α5-containing $GABA_A$ receptors are thus currently developed as cognition enhancers. In addition, Istvan Mody recently demonstrated that such compounds could also be beneficial for reducing brain damage after stroke[66].

This scheme of actions of different $GABA_A$ receptor subtypes for sure is oversimplified. One cannot assume that a single receptor subtype is solely

[62] Löw K, Crestani F, Keist R, Benke D, Brünig I, Benson JA, Fritschy JM, Rülicke T, Bluethmann H, Möhler H, Rudolph U (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. *Science* 290, 131-134.

[63] Möhler H (2011) The rise of a new GABA pharmacology. *Neuropharmacol* 60, 1042-1049.

[64] Knabl J, Witschi R, Hösl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Möhler H, Zeilhofer HU (2008) Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes. *Nature* 451, 330-334.

[65] Rudolph U, and Möhler H (2014) $GABA_A$ receptor subtypes: therapeutic potential in Down syndrome, affective disorders, schizophrenia, and autism. *Annu Rev Pharmacol Toxicol* 54, 483-507.

[66] Clarkson AN, Huang BS, Macisaac SE, Mody I, Carmichael ST (2010) Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke. *Nature* 468, 305-309.

responsible for the anxiolytic action of diazepam and for sure, other receptor subtypes contribute to this action, and this has also been demonstrated in the last couple of years. Nevertheless, the receptor types indicated represent promising targets for eliciting the respective actions and it is thus worthwhile to develop appropriate receptor subtype-selective compounds.

This was one of the reasons we started a collaboration with Jim Cook, University Wisconsin, Milwaukee, USA in 2002. Jim Cook concentrates on the synthesis of classical benzodiazepines and imidazobenzodiazepines to make them more $GABA_A$ receptor subtype-selective. Due to the well-known extremely low toxicity of these compound classes, the risks of their failure in a clinical application are low. By investigating quite a few of the compounds synthesized by Jim Cook and his group, during the last 12 years we have identified and helped to develop the compound HZ 166, that is a positive allosteric modulator highly selective for $\alpha2\beta\gamma2/\alpha3\beta\gamma2$ receptors[67] *(Fig 5A)*. It has been demonstrated to exhibit anxiolytic and much reduced sedative effects and is also active against neuropathic pain[68,69]. Another compound, PWZ-029, is one of the most selective negative allosteric modulators at $\alpha5\beta\gamma2$ receptors, and as expected, it enhances cognition[70] *(Fig 5B)*.

[67] Rivas FM, Stables JP, Murphree L, Edwankar RV, Edwankar CR, Huang S, Jain HD, Zhou H, Majumder S, Sankar S, Roth BL, Ramerstorfer J, Furtmüller R, Sieghart W, Cook JM (2009) Antiseizure activity of novel gamma-aminobutyric acid(A) receptor subtype-selective benzodiazepine analogues in mice and rat models. *J Med Chem* 52, 1795-1798.

[68] Savic MM, Majumder S, Huang SM, Edwankar RV, Furtmüller R, Joksimovic S, Clayton T, Ramerstorfer J, Milinkovic MM, Roth BL, Sieghart W, and Cook JM (2010) Novel positive allosteric modulators of $GABA_A$ receptors: Do subtle differences in activity at α1 plus α5 versus α2 plus α3 subunits account for dissimilarities in behavioural effects in rats? *Progr Neuro-Psychopharmacol Biol Psychiatry* 34, 376-386.

[69] Di Lio A, Benke D, Besson M, Desmeules J, Daali Y, Wang ZJ, Edwankar R, Cook JM, Zeilhofer HU (2011) HZ 166, a novel $GABA_A$ receptor subtype-selective benzodiazepine site ligand, is antihyperalgesic in mouse models of inflammatory and neuropathic pain. *Neuropharmacol* 60, 626-632.

[70] Savic MM, Clayton T, Furtmüller R, Gavrilovic I, Samardzic J, Savic S, Huck S, Sieghart W, Cook JM (2008) PWZ-029, a compound with moderate inverse agonist functional selectivity

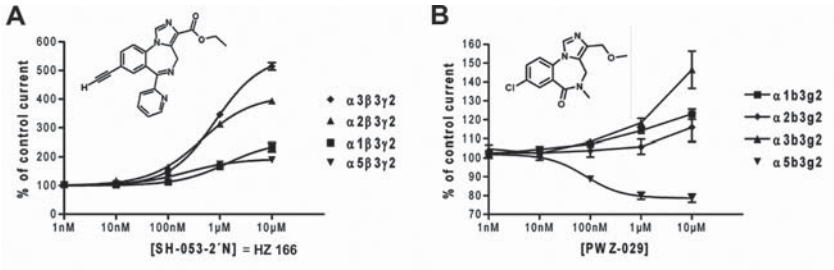

Figure 5: (A) Structural formula of the benzodiazepine HZ 166 and concentration-response curves of this compound at various recombinant receptor subtypes expressed in *Xenopus laevis* oocytes and measured by the two electrode voltage clamp technique. Data are from Ref 67.
(B) Structural formula of the imidazobenzodiazepine PWZ-029 and concentration-response curves of this compound at various recombinant receptor subtypes expressed in *Xenopus laevis* oocytes and measured by the two electrode voltage clamp technique. Data are from Ref 70.

Recently, it has been demonstrated that airway smooth muscle cells contain only a restricted repertoire of $GABA_A$ receptor subunits, and that activating α4 or α5 subunit-containing receptors facilitates relaxation and reduces mucus formation[71,72], suggesting a novel therapeutic option for patients with severe bronchospasm. By investigating the action of a long series of Jim Cook´s compounds at various recombinant $GABA_A$ receptor subtypes we for the first time developed positive allosteric modulators that are highly selective for α4βγ2 or α5βγ2 receptors and that are active in preventing mucus formation in bronchia and might have a possible application for the treatment of asthma (Emala, unpublished results). In addition, recently it has been demonstrated

at $GABA_A$ receptors containing alpha5 subunits, improves passive, but not active avoidance learning in rats. *Brain Res* 1208, 150-159.

[71] Mizuta K, Xu D, Pan Y, Comas G, Sonett JR, Zhang Y, Panettieri RA Jr, Yang J, Emala CW Sr (2008) $GABA_A$ receptors are expressed and facilitate relaxation in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol 294, L1206-1216.*

[72] Gallos G, Yim P, Chang S Zhang Y, XuD, Cook JM, Gerthoffer WT, Emala CW Sr (2012) Targeting the restricted α-subunit repertoire of airway smooth muscle $GABA_A$ receptors augments airway smooth muscle relaxation. *Am J Physiol Lung Cell Mol Physiol* 302, L248-256.

that highly specific and potent α5-$GABA_A$ receptor agonists negatively regulate the very aggressive, MYC-driven, "group3" medulloblastomas[73]. We are continuing to investigate such compounds to identify novel treatment principles and to possibly develop clinically important drugs.

In the last couple of years we have investigated whether the extracellular α+β- interface of $GABA_A$ receptors might also function as a drug binding site. Since this interface is homologous to the benzodiazepine binding site of $GABA_A$ receptors located at the α+γ2- interface *(Fig. 3A)*, we argued that at least some of the benzodiazepine site ligands might also be able to bind at the α+β- interface. To avoid binding to the benzodiazepine binding site, receptors had to be investigated that were composed of α1 and β3 subunits, only. Such receptors that have recently been identified in the brain seem to be composed of 2 α1 and 3 β3 subunits, and thus, contain two GABA binding sites at the β3+α1- interface, two α1+β3- interfaces and one β3+β3- interface. In a screen of >100 benzodiazepine site ligands, the pyrazoloquinolinone CGS 9895 *(Fig. 6A)*, which had been demonstrated previously to interact with the benzodiazepine binding site of $GABA_A$ receptors with high affinity, was identified to strongly modulate α1β3 $GABA_A$ receptors. To investigate whether this effect was mediated via the extracellular α1+β3- interface, a steric hindrance approach was applied. Several amino acid residues known to be located within the benzodiazepine binding site at the α1+ side, as well as β3- residues homologous to residues located at the γ2- interface within the benzodiazepine binding site, were mutated to cysteines. Mutations that did not significantly change the properties of the receptor (GABA-induced current, CGS 9895 modulation) were then reacted with MTSEA-biotin, a cysteine reactive reagent, and

[73] Sengupta S, Weeraratne SD, Sun H, Phallen J, Rallapalli SK, Teider N, Kosaras B, Amani V, Pierre-Francois J, Tang Y, Nguyen B, Yu F, Schubert S, Balansay B, Mathios D, Lechpammer M, Archer TC, Tran P, Reimer RJ, Cook JM, Lim M, Jensen FE, Pomeroy SL, Cho YJ (2013) α5-$GABA_A$ receptors negatively regulate MYC-amplified medulloblastoma growth. *Acta Neuropathol* 127, 593-603.

possible changes in the effects of CGS 9895 were investigated. Covalent binding of MTSEA-biotin within the drug binding site should have prevented access of the drug to the receptor and by that, should have prevented the drug effect. This was actually the case, indicating that CGS 9895 mediates its effect via the extracellular α1+β3- interface[74]. In other experiments using α1β3γ2 receptors, CGS 9895 did not modulate GABA-induced currents at nM concentrations but at microM concentrations elicited a comparable current enhancement in α1β3 and α1β3γ2 receptors. The effects of CGS 9895 on α1β3γ2 receptors in contrast to that of diazepam could not be inhibited by the benzodiazepine site antagonist Ro15-1788, and 50 nM CGS 9895, a concentration that completely saturates the high affinity benzodiazepine binding site of $GABA_A$ receptors,

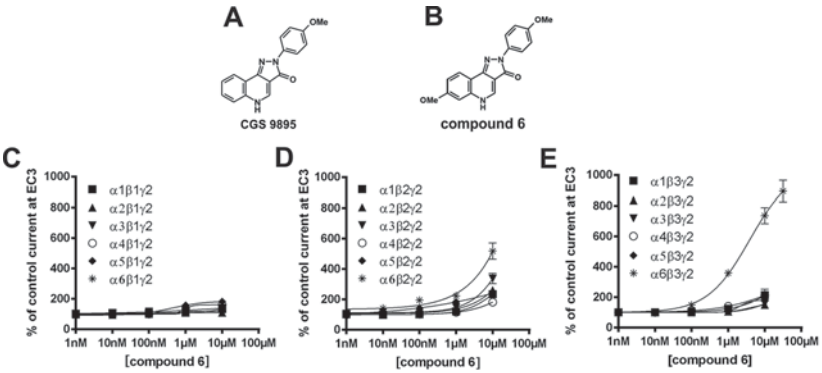

Figure 6: (A) Structural formula of the pyrazoloquinolinone CGS 9895. (B) Structural formula of the pyrazoloquinolinone compound 6. (C) Concentration-response curves of compound 6 at various recombinant $GABA_A$ receptor subtypes containing β1 subunits and expressed in *Xenopus laevis* oocytes and measured by the two electrode voltage clamp technique. (D) Concentration-response curves of compound 6 at various recombinant $GABA_A$ receptor subtypes containing β2 subunits.(E) Concentration-response curves of compound 6 at various recombinant $GABA_A$ receptor subtypes containing β3 subunits. Data are from Ref 77.

[74] Ramerstorfer J, Furtmüller R, Sarto-Jackson I, Varagic Z, Sieghart W, Ernst M (2011) The $GABA_A$ receptor α+β- interface: a novel target for subtype-selective drugs. *J Neurosci* 31, 870-877.

but does not stimulate GABA-induced currents, was able to completely inhibit the effects of diazepam at α1β3γ2 receptors. These data indicate that CGS 9895 is an antagonist (null modulator) at the high affinity benzodiazepine binding site at nM concentration and stimulates the receptor at microM concentrations via the α1+β3- interface[74].

What is the importance of this novel binding site? Drugs interacting with the α+β- site of $GABA_A$ receptors exhibit actions similar to benzodiazepines. They cannot directly activate $GABA_A$ receptors but can only allosterically modulate ongoing GABA-induced currents. Such drugs, thus, will exhibit low toxicity, similar to the benzodiazepines. Whereas benzodiazepines require the presence of a γ subunit for their action, an α+β- interface is present not only at αβγ, but also at αβ, αβδ, αβε, αβθ, αβπ receptors. Drugs indiscriminately interacting with all possible α+β- sites will thus exhibit a much broader action than the benzodiazepines and might be excellent anticonvulsants. However, drugs that selectively interact with a specific α(1–6)+β(1-3)- interface can address novel receptor subtypes. Whereas benzodiazepines predominantly interact with α(1–6)βγ2 receptors irrespective of the type of β subunit present in the receptor, α+β- site ligands can differentiate between α(1–6)βγ2 receptors containing either β1, β2, or β3 subunits. Recent evidence indicates that αβγ2 receptors containing β1 subunits might mediate the sedative actions of benzodiazepines. Drugs activating any one of the α(1–6)β(2/3)γ2 receptors but avoiding modulation of αβ1γ2 receptors might thus combine the beneficial actions of benzodiazepines with a loss of their sedative actions. Drugs interacting with the α+β- site of $GABA_A$ receptors will thus have novel and possibly important actions[75].

[75] Sieghart W, Ramerstorfer J, Sarto-Jackson I, Varagic Z, Ernst M (2012) A novel $GABA_A$ receptor pharmacology: drugs interacting with the α+β- interface. *Br J Pharmacol* 166, 476-485.

In the meantime, we have investigated >25 pyrazoloquinolinones and studied their receptor subtype selectivity[76,77]. Interestingly, one of these compounds is the first highly selective positive allosteric modulator of α6β3γ2 receptors, thus providing the proof of principle for the assumption that subtype-selective compounds acting via the α+β- site can be generated *(Fig 6B)*. Compound 6 does not interact with α6β1γ2 receptors and only weakly interacts with α6β2γ2 receptors *(Fig 6C-E)*, thus proving the concept that α+β- site drugs can distinguish between receptors containing different β subunit types. Previously known compounds exhibiting some selectively for α6-containing $GABA_A$ receptors, such as the diuretics furosemide[78] or amiloride[79], or the dissociative anesthetic and NMDA receptor blocker ketamine[80], are difficult to be used in animal experiments or man because of their additional actions at non-GABAergic systems. Compound 6 is currently used for the investigation of the function of α6 receptors in the brain. Recently, we have identified highly interesting effects of this compound in an animal model of a human disease and the application of this compound for the treatment of this disease is the topic of a current patent application.

[76] Varagic Z, Wimmer L, Schnürch M, Mihovilovic MD, Huang S. Rallapalli S, Cook JM, Mirheydari P, Ecker GF, Sieghart W, Ernst M (2013) Identification of novel positive allosteric modulators and null modulators at the $GABA_A$ receptor α+β- interface. *Br J Pharmacol* 169, 371-383.

[77] Varagic Z, Ramerstorfer J, Huang S, Rallapalli S, Sarto-Jackson I, Cook J, Sieghart W, Ernst M (2013) Subtype selectivity of α+β- site ligands of $GABA_A$ receptors - identification of the first highly specific positive modulators at α6β2/3γ2 receptors. *Br J Pharmacol 169, 384-399.*

[78] Wafford KA, Thompson SA, Thomas D, Sikela J, Wilcox AS, Whiting PJ (1996) Functional characterization of human gamma-aminobutyric acidA receptors containing the alpha4 subunit. *Mol Pharmacol* 50, 670-678.

[79] Drafts BC, Fisher JL (2004) Structural determinants of the pharmacological properties of the $GABA_A$ receptor alpha6 subunit. *J Pharmacol Exp Ther* 309, 1108-1115.

[80] Hevers W, Hadley SH, Lüddens H, Amin J (2008) Ketamine, but not phencyclidine, selectively modulates cerebellar $GABA_A$ receptors containing alpha6 and delta subunits. *J Neurosci* 28, 5383-5393.

In summary, I have provided an overview on selected topics of our work performed during 35 years of $GABA_A$ receptor research. This work started with the [^{3}H]flunitrazepam photolabeling experiments of brain membranes leading to the first identification of distinct $GABA_A$-benzodiazepine receptors in the brain. Generation of $GABA_A$ receptor subunit-selective antibodies then allowed to determine the subunit-composition of $GABA_A$ receptor subtypes and the heterogeneity of $GABA_A$ receptors turned out to be much larger than expected. The antibodies, however, were also used for the investigation of the regional, cellular and subcellular distribution of the subunits in the brain and thus, provided most of our current knowledge on $GABA_A$ receptor heterogeneity and location[81]. To identify receptors in situ and to determine their function in the brain, a careful pharmacological characterization of the receptors and the identification of drugs selectively interacting with receptor subtypes are essential. Our work during the last couple of years has identified compounds that are highly selective for $\alpha 1\beta\gamma 2$, $\alpha 2/3\beta\gamma 2$, $\alpha 4\beta\gamma 2$, $\alpha 5\beta\gamma 2$, or $\alpha 6\beta\gamma 2$ receptors. These compounds can now be used for investigating the function of the respective receptors in the brain and for studying their effects in various animal models of human diseases of the brain and of the periphery. It is clear now, that $GABA_A$ receptor subtypes can modulate anxiety, cognition, sleep, pain, epilepsy, stroke, schizophrenia, depression, and autism, as well as asthma, tumors, and possibly also diabetes. Drugs selectively modulating the receptor subtypes suitable for treating these diseases will thus become important for a variety of clinical applications. Our identification and location of multiple drug binding sites in $GABA_A$ receptors combined with our modeling and docking studies opens the route for structure-based drug design and will accelerate the development of novel receptor subtype-selective drugs.

[81] Olsen RW and Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of γ-Aminobutyric $Acid_A$ Receptors: Classification on the Basis of Subunit Composition, Pharmacology, and Function–Update. *Pharmacol Rev* 60: 243-260.

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