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Investigation of new $GABA_A$ receptor ligands for their receptor subtype selectivity

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CONTENTS

I ABSTRACT

$GABA_A$ receptors are the most important inhibitory neurotransmitter receptors in the mammalian brain. The existence of a variety of receptor subtypes in combination with their distinct distribution in the brain as well as the presence of many different drug binding sites at each receptor subtype results in an extremely complex $GABA_A$ receptor pharmacology. Drugs such as benzodiazepines, barbiturates, neuroactive steroids, anticonvulsants and anesthetics have been identified as modulators of $GABA_A$ receptors. Their clinical and pharmacological effects allow conclusions on the function of these receptors in the brain. Thus, classical benzodiazepines such as diazepam, bind to the allosteric benzodiazepine binding site of some $GABA_A$ receptors and produce anxiolytic, anticonvulsant, sedative and muscle relaxant effects. Receptors containing different α subunits of $\alpha\beta\gamma$ receptors seem to mediate different pharmacological effects evoked by diazepam.

In this master thesis I investigated the effects of 12 newly synthesized benzodiazepines at $GABA_A$ receptors composed of $\alpha\beta\gamma$ subunits. The aim of this study was to determine a possible receptor subtype selectivity of these compounds as well as to investigate whether a structure-activity relationship could be established. For that, different recombinant $GABA_A$ receptor subtypes were expressed in *Xenopus laevis* oocytes and the effects of these compounds on GABA-induced currents were measured using the two-electrode voltage clamp technique.

All of the compounds were able to modulate GABA-induced currents at some receptor subtypes, but most of them showed similar effects at all receptor subtypes investigated. Four compounds, however, exhibited some receptor subtype selectivity by excerting differential effects depending on the α subunit present within the receptor. In addition, these measurements clearly identified structural features that enhance or reduce the potency and efficacy of these compounds at the benzodiazepine binding site of $GABA_A$ receptors. Knowledge of the structure-activity relationship could be used to create new ligands for this binding site. Compounds that are selectively interacting with receptors containing specific α subunits can become an alternative to conventional benzodiazepine site ligands.

II ZUSAMMENFASSUNG

$GABA_A$ Rezeptoren sind die wichtigsten inhibitorischen Neurotransmitter Rezeptoren im Säugetiergehirn. Die große Anzahl von $GABA_A$ Rezeptor-Subtypen, ihre unterschiedliche Verteilung im Gehirn, sowie die Existenz verschiedenster Bindungsstellen an jedem einzelnen Rezeptor-Subtyp erklären die komplexe Pharmakologie der $GABA_A$ Rezeptoren. Zahlreiche klinisch und pharmakologisch relevante Wirkstoffe wie etwa Benzodiazepine, Barbiturate, neuroaktive Steroide, Konvulsiva und Anästhetika konnten als Modulatoren des $GABA_A$ Rezeptors identifiziert werden. Klassische Benzodiazepine wie Diazepam, binden an eine spezifische allosterische Benzodiazepin-Bindungsstelle am Rezeptor und haben angstlösende, krampflösende, sedative und muskelrelaxierende Wirkungen. Die unterschiedlichen pharmakologischen Effekte von Diazepam werden über $\alpha\beta\gamma$ Rezeptoren mit unterschiedlichen α Untereinheiten ausgelöst.

In dieser Masterarbeit wurden die Effekte von 12 neu synthetisierten Benzodiazepinen auf $\alpha\beta\gamma$ $GABA_A$ Rezeptoren untersucht. Das Ziel dieser Arbeit war, die Substanzen auf Subtyp-selektive Wirkungen zu testen und einen möglichen Zusammenhang zwischen chemischer Struktur und Aktivität zu analysieren. Dafür wurden unterschiedliche rekombinante $GABA_A$ Rezeptoren in *Xenopus laevis* Oozyten exprimiert und die Wirkung dieser 12 Substanzen auf die GABA-induzierten Ströme mittels der „Two-Elektrode Voltage Clamp“ Methode gemessen.

Alle untersuchten Substanzen waren in der Lage, GABA-induzierte Ströme in zumindest einigen Rezeptor-Subtypen zu modulieren, jedoch zeigten die meisten ähnliche Effekte in allen untersuchten Rezeptor-Subtypen. Vier Substanzen hatten jedoch eine gewisse Rezeptor-Subtyp Selektivität, da ihre Effekte in Subtypen mit unterschiedlicher α Untereinheit unterschiedlich stark waren. Außerdem konnte ein Zusammenhang zwischen chemischer Struktur und erhöhter oder reduzierten Affinität oder Potenzierung an der Benzodiazepin-Bindungsstelle festgestellt werden. Informationen über Struktur-Wirkungs-Beziehungen können dazu verwendet werden, um neue Liganden für diese Bindungsstelle zu synthetisieren. Selektiv wirkende Substanzen könnten eine Alternative zu konventionellen Benzodiazepin-Bindungsstellen Liganden werden.

1 INTRODUCTION

1.1 GABA

GABA (γ-aminobutyric acid) is an important inhibitory neurotransmitter in the central nervous system and binds to GABA receptors (Sieghart, 1995). It is synthesized by decarboxylation of glutamic acid, also called glutamate, by the enzyme glutamic acid decarboxylase (GAD) (Bear, 2001) and afterwards stored in vesicles of the presynaptic terminals of neurons.

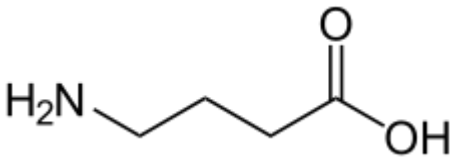

Fig.1: Chemical structure of GABA

1.2 GABAergic transmission

Between 20-50% of the central synapses use GABA as their primary neurotransmitter (Sivilotti and Nistri, 1991; Hevers and Luddens, 1998). GABAergic synapses which are located in many brain regions like cerebellum, brainstem, thalamus, hippocampus, cerebral cortex and basal ganglia, are of particular importance for neuronal activity and information processing (Minelli et al., 1995).

Ion channels play a crucial role in signal transduction. Arriving nerve impulses primarily trigger an intracellular Ca^{2+} increase via voltage gated calcium channels. Ca^{2+} then induces fusion between the vesicles and the plasma membrane and the neurotransmitter GABA is released from the presynaptic terminal of the neuron into the synaptic cleft. *In vivo* there is a GABA concentration of up to 3 mM in the synaptic cleft for a very short time after release of GABA (Mozrzymas et al., 2003; Glykys and Mody, 2007). GABA diffuses to the post-synaptic membrane where it binds to GABA receptors (Kash et al., 2004), in most cases leading to inhibitory effects at the postsynaptic neuron and therefore, to a decreased neuronal excitability.

However, under some special conditions, GABA can also increase neuronal excitability, for example in early development (Ben-Ari, 2002), in the case of diseases, or when the chloride concentration within the cell is increased and therefore, the chloride equilibrium potential is changed (Olsen and Sieghart 2008).

After binding to the GABA receptors, GABA is removed immediately from the synaptic cleft by GABA transporters at the presynaptic terminal or at surrounding glia cells (Iversen and Kelly, 1975) and is then either recycled in vesicles in the presynaptic neuron or catabolized to succinate semialdehyde by GABA transaminases to be used in the citric acid cycle (Minelli et al., 1995).

1.3 GABA receptors

There are two types of GABA receptors: on the one hand, the $GABA_A$ receptor which is a fast ligand-gated ion channel and on the other hand, the metabotropic $GABA_B$ receptor which is coupled to Ca^{2+} and K^+ channels via G-proteins and additionally modulating second messenger systems (Wojcik and Neff, 1984; Bormann, 1988). In the literature, sometimes a third GABA receptor class, the $GABA_{C,}$ is found, but due to the fact that it is structurally and functionally related to $GABA_A$ receptors, it belongs to the class of $GABA_A$ receptors (Sieghart, 1995). Nevertheless, these receptors show a different pharmacological pattern, because they are insensitive to most of the $GABA_A$ receptor agonists and modulators (Olsen and Sieghart, 2008). At least some of these receptors seem to be homopentameric chloride channels consisting of one of the three existing ρ subunits.

The $GABA_A$ receptor is a chloride ion channel that can be activated and opened by GABA. Binding of GABA to these receptors leads to conformational changes of the receptor, the neuronal membrane conductance for chloride ions increases, and under normal circumstances, chloride ions are forced through the channel into the cell due to the high chloride concentration in the extracellular space and increase the intracellular chloride level. The hyperpolarization of the membrane reduces the probability to initiate an action potential and thus, is leading to neuronal inhibition (Sivilotti and Nistri, 1991; Olsen and Sieghart, 2008).

$GABA_B$ receptors are G-protein coupled receptors. G-protein mediated intracellular mechanisms elicit an enhancement of the K^+ conductance or a decrease of the Ca^{2+} conductance, and furthermore, they cause an inhibition of cAMP production. As a result, on the one hand, $GABA_B$ receptor activation leads to inhibition of neurotransmitter release at the presynaptic side due to closing of Ca^{2+} channels and on the other hand, activation of K^+ channels at the postsynaptic side leads to hyperpolarization and therefore, action potentials are prevented from firing (Bowery et al., 2002). Only a limited number of pharmacological tools have been discovered so far that are interacting with this receptor type, for example the selective agonist baclofen.

1.4 $GABA_A$ receptors

1.4.1 Structure of $GABA_A$ receptors

$GABA_A$ receptors are members of the cys-loop receptor superfamily that in addition to these receptors comprise the nicotinic acetylcholine receptors (Corringer et al., 2000), ionotropic serotonin type 3 receptors (Davies et al., 1999), Zn^{2+} activated ion channels (Davies et al., 2003) and the glycine receptors (Breitinger and Becker, 2002; Kirsch, 2006). There exist also a range of invertebrate cys-loop receptors like the acetylcholine binding protein (Brejc et al., 2001) and the glutamate-gated chloride channels (Cully et al., 1994), as well as histamine-gated chloride channels and serotonin-gated chloride channels (Thompson et al., 2010; Zhang et al., 2013) and furthermore, related proteins in prokaryotes could be identified (Tasneem et al., 2005).

$GABA_A$ receptors are ligand-gated ion channels consisting of five subunits. Each subunit is composed of a long extracellular N-terminal domain containing the ligand binding sites as well as a cys-loop, followed by four transmembrane (TM) segments, and end with a short extracellular C-terminus. The TM2 domains of the five subunits are forming an ion channel through the membrane (Keramidas et al., 2004).

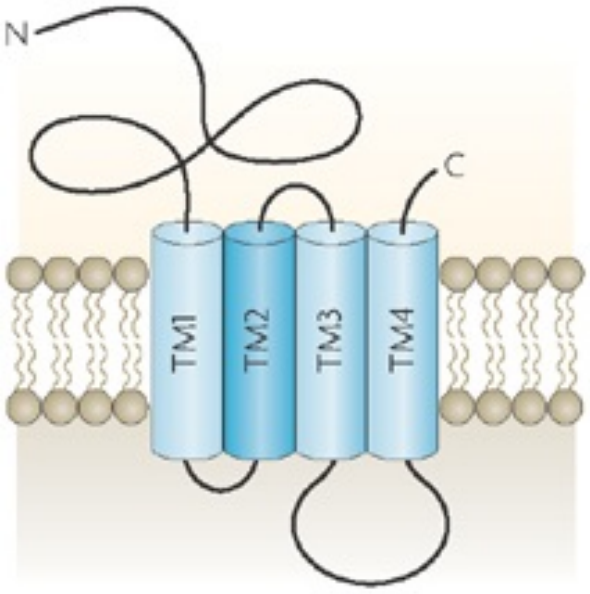

Fig.2: Schematic structure of a subunit from the cys-loop pentameric ligand-gated ion channel superfamily. The subunit is organized in an extracellular N-terminal domain, four transmembrane α helix domains (TM1-4) and a short C-terminal domain. The figure is taken from (Jacob et al., 2008).

The following 19 $GABA_A$ receptor subunits have been identified in mammalian brain: 6 α, 3 β, 3 γ, 1 δ, 1 ε, 1 π, 1 θ and 3 ρ. Given this variety of subunits, a large number of different subunit combinations can be formed resulting in distinct $GABA_A$ receptor subtypes, but most of the $GABA_A$ receptors are built out of two α, two β and one γ, δ, or ε subunit (Sieghart and Sperk, 2002).

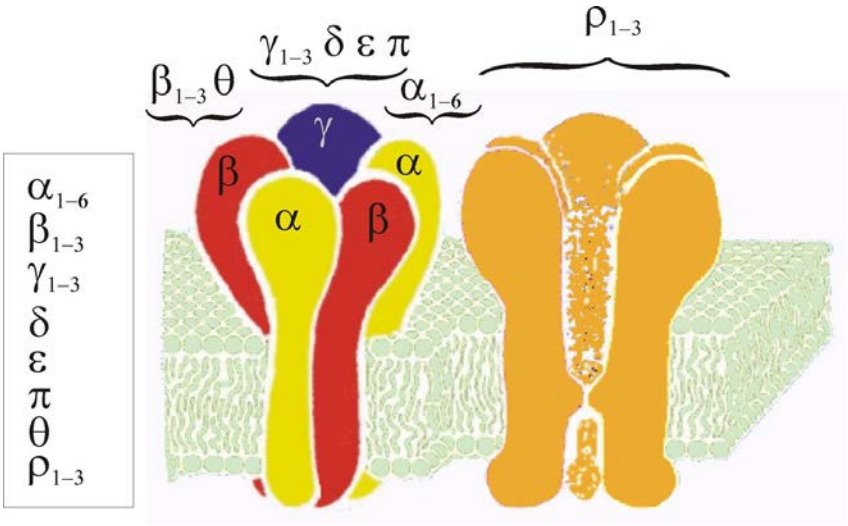

Fig.3: $GABA_A$ receptors consisting of different subunit classes. A variety of subunits can be combined to different receptor subtypes. The majority of $GABA_A$ receptors consist of 2α, 2β and 1γ subunit, the type of α, β and γ can vary (α1-6, β1-3, γ1-3). The ρ subunits can form homopentamers and have been referred as $GABA_C$ receptors. This figure was modified from (Olsen and Sieghart, 2008).

Each subunit has assigned a plus and a minus side. The five subunits forming $GABA_A$ receptors give rise to five interfaces. In the extracellular domain of αβγ receptors there are two GABA binding sites located at the β+ and the α- contact site (Smith and Olsen, 1995).

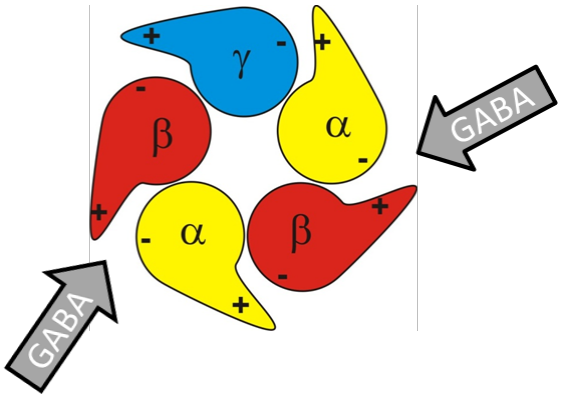

Fig.4: Schematic structure of the extracellular domain of αβγ $GABA_A$ receptors (top view). Each subunit has a plus and a minus side, one plus and one minus side are forming an interface. The grey arrows indicate the location of the two GABA binding sites at the β+α- interface.

1.4.2 Distribution

$GABA_A$ receptors are expressed all over the central nervous system with a different assemblage and exist in all organisms which have a nervous system including invertebrates (Buckingham et al., 2005). In addition, they even occur in non-neural tissues like the pancreas (Borboni et al., 1994). The different $GABA_A$ receptor subunits exhibit a distinct but overlapping cellular and regional distribution in the central nervous system.

The most abundant subunits expressed in the brain are the α1 in combination with β2 and γ2 subunits (Sieghart and Sperk, 2002). With knock-out studies it was demonstrated that the loss of the α1 subunit in mice leads to a 50% reduction of all $GABA_A$ receptors (Sur et al., 2001). The α2 and α3 subunits are preferentially located in cortical regions and amygdala and are lower expressed than the α1 subunit. The α4 containing receptors are predominantly located in the thalamus and basal ganglia (Pirker et al., 2000). The α5 subunits occur only rarely and mainly in hippocampus (Pirker et al., 2000) and α6 subunits are only located in the granule cell layer of cerebellum and the cochlear nucleus (D'Hulst et al., 2009). The β2 is the most highly expressed β subunit (Sur et al., 2001) and γ2 has the highest expression level among γ subunits and is the most frequently occurring subunit since it is incorporated in up to 75% of all $GABA_A$ receptors in the brain (Sieghart and Ernst, 2005).

$GABA_A$ receptors are not only present at the synapse but are also found extrasynaptically. Extrasynaptic receptors exhibit a higher sensitivity to GABA than synaptic receptors and this is required due to the lower GABA concentration (300 nM to 3 µM) (D'Hulst et al., 2009) occurring around these receptors. For synaptic localization the γ2 subunit is needed to be integrated into the receptor, mostly in combination with α1, α2 or α3 subunits (Kasugai et al., 2010). The majority of α4, α5 and α6 combinations as well as receptors containing the δ or ε subunits are located extrasynaptically (Nusser et al., 1998). The δ subunit is often combined with α4 and α6 subunits (Barrera et al., 2008). The localization of ε, θ and π subunits is not well known (Sieghart and Sperk, 2002).

1.4.3 $GABA_A$ receptor binding sites and pharmacology

As the activity of the receptor is influenced by a plurality of pharmacologically and clinically important drugs such as benzodiazepines, barbiturates, neuroactive steroids, anticonvulsants and anesthetics, it opens a large spectrum for medical treatment. Most of these drugs act by binding to allosteric sites at $GABA_A$ receptors and so modulate GABA-induced chloride flux.

In the extracellular domain of $GABA_A$ receptors five interfaces arise between the subunits creating different binding sites. The variety of binding sites at a single receptor subtype in combination with a distinct distribution of these receptors in the central nervous system explains the complexity of the $GABA_A$ receptor pharmacology (Ernst et al., 2005). At the two GABA binding interfaces ($\beta+/\alpha-$), besides GABA some other receptor agonists/antagonists are able to bind, for instance the agonist muscimol opens the channel, whereas bicuculline inhibits the $GABA_A$ receptor (Sieghart, 1995). In $\alpha\beta\gamma$ receptors, the $\alpha+/\gamma-$ interface forms the benzodiazepine (BZ) binding site (Sigel and Buhr, 1997; Ernst et al., 2003). Agonists or positive allosteric modulators at the BZ site like classical benzodiazepines enhance the ongoing GABA-induced currents and by that exhibit muscle relaxant and sedative properties and play a role in anxiety, learning and memory (Sieghart, 1995; Sigel and Buhr, 1997). On the contrary, inverse agonists (negative allosteric modulators) produce opposite effects, they have anxiogenic, stimulant and proconvulsant properties by reducing GABA-induced currents (D'Hulst et al., 2009). Antagonists or null modulators, however, block the BZ binding site, and thereby inhibit the binding of agonists or inverse agonists at the BZ site, without influencing GABA-induced currents and as a result, reduce or completely inhibit the action of these drugs (Sieghart, 1995). Flumazenil (Ro 15-1788) acts as an antagonist at the BZ binding site. Between the $\alpha+$ and $\beta-$ side the so called CGS binding site is located. Compounds interacting with this binding site have much more targets than benzodiazepines, because they can bind to $\alpha\beta$, $\alpha\beta\gamma$, $\alpha\beta\delta$, $\alpha\beta\varepsilon$ and $\alpha\beta\pi$ $GABA_A$ receptors. The CGS 9895 compound was identified as an $\alpha+\beta-$ site agonist (Ramerstorfer et al., 2011). So far, no drug has been identified that binds at the fifth interface between the $\gamma+$ and $\beta-$ side.

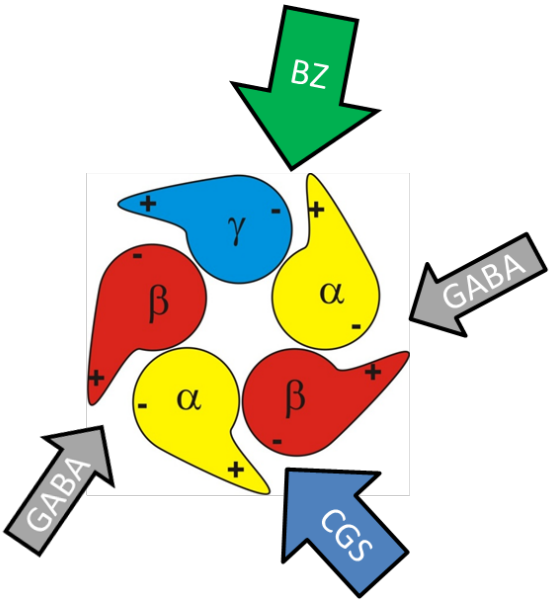

Fig.5: Schematic structure of the extracellular domain of αβγ $GABA_A$ receptors indicating the location of the binding sites (top view). The green arrow indicates the location of the benzodiazepine (BZ) binding site at the α+/γ- interface, the two GABA binding sites are shown by the grey arrows at the β+/γ- interface, and the recently identified CGS site is located at the α+/β- interface (blue arrow).

Finally, steroids, anesthetics and barbiturates are able to bind at the trans-membrane domain of the receptors and by that, modulate receptor activity. These compounds enhance GABA-induced currents at low concentrations, and in contrast to benzodiazepines and CGS 9895, in addition to their allosteric modulatory properties, can also directly activate $GABA_A$ receptors at higher concentrations (Sieghart et al., 2012), thus, exhibiting much more toxicity than the benzo-diazepines at overdoses (Bonin and Orser, 2008).

Deficits in $GABA_A$ receptor expression can be associated with epilepsy, cognitive deficits, schizophrenia, anxiety disorder, depression (Jacob et al., 2008).

1.4.4 Benzodiazepines

Benzodiazepines have been discovered in 1957 and in spite of their high activity they are known as safe drugs in terms of application due to their low toxicity. They can only modulate ongoing GABAergic currents, but are not able to activate the receptor in the absence of GABA (Macdonald and Olsen, 1994; Mohler

et al., 2001). Classical benzodiazepines like diazepam or flunitrazepam bind to the allosteric BZ binding site of the $GABA_A$ receptor. The α and γ subunits are needed to form the BZ binding site and thus, affect the pharmacology of benzodiazepines. In contrast, the β subunit does not influence the effects of benzodiazepines at $GABA_A$ receptors (Hadingham et al., 1993). Benzodiazepines mainly interact with $\alpha1\beta\gamma2$, $\alpha2\beta\gamma2$, $\alpha3\beta\gamma2$, $\alpha5\beta\gamma2$ receptors (Richter et al., 2012) but exhibit smaller effects at $\gamma1$ and $\gamma3$ containing receptors (Khom et al., 2006). They do not interact with $\alpha4\beta\gamma2$ and $\alpha6\beta\gamma2$ receptors (Barnard, 1998) with the exception of some imidazobenzodiazepines like flumazenil (Ro 15-1788) or bretazenil (Olsen and Sieghart, 2008). However, imidazobenzodiazepines with a pendant phenylring are not able to bind at the diazepam insensitive ($\alpha4$, $\alpha6$) receptors.

1.4.5 Diazepam and subtype selectivity

Diazepam, also known as Valium, is a classical benzodiazepine and although it can lead to addiction after 3-6 weeks, it is in clinical use since 1963. Diazepam was used to investigate the functions of $\alpha\beta\gamma$ $GABA_A$ receptors containing different α subunits. Single α subunits were mutated and by that, the respective $GABA_A$ receptors became diazepam-insensitive. Wild-type mice containing the original diazepam sensitive receptors and mutated mice containing the diazepam-insensitive receptors instead were treated with diazepam and afterwards the effects were compared (Rudolph and Mohler, 2004; Atack, 2005; Whiting, 2006). Due to the fact that diazepam cannot bind to the mutated receptors, the missing diazepam effects in mutated mice provided information on diazepam-induced pharmacological and behavioral responses associated with the mutated receptors. It could be shown that $\alpha\beta\gamma$ receptors containing different α subunits seem to mediate different pharmacological effects evoked by diazepam (Rudolph and Mohler, 2004). Receptors containing the $\alpha1$ subunit trigger sedative and anticonvulsive effects after stimulation by diazepam (Rudolph et al., 1999; McKernan et al., 2000). Receptors containing $\alpha2$ and $\alpha3$ subunits appear to be responsible for anxiolytic and muscle relaxant effects, in addition, they mediate analgetic actions. The $\alpha2$ containing receptors are the most important anxiolytic receptors, $\alpha3$ containing receptors are not needed for anxiolytic actions but are participating (Whiting, 2003; Mohler et al., 2005; Mohler, 2007). At $\alpha\beta\gamma$ receptors

containing the α5 subunit diazepam has effects on learning and memory. In α5 subunit knock-out mice, the hippocampus dependent associative learning is increased (Crestani et al., 2002) and the spatial memory in a water maze model is improved (Collinson et al., 2002). It is assumed that an α5 subunit selective inverse agonist may increase hippocampally based cognitive functions (D'Hulst et al., 2009). As expected, diazepam shows no effects at α4 and α6 containing receptors because these receptors are diazepam insensitive (Barnard et al., 1998).

In the last years, there was a search for subtype selective drugs interacting with the BZ binding site of $GABA_A$ receptors because drugs with subtype selective action are expected to have more specific effects and less side effects and in addition, they can help to define receptor subtype functions. So far, some BZ binding site ligands can distinguish between receptor subtypes which contain specific α subunits (Barnard et al., 1998) and thus, evoke different pharmacological patterns. A positive allosteric modulator at α2 or α3 containing receptors has been identified to inhibit inflammatory and neuropathic pain (Knabl et al., 2008). Furthermore, α3 selective agonists could be used for the treatment for sensor motoric gating deficits in psychiatric condition (schizophrenia) (Lewis et al., 2008).

1.5 Aim of the study

In this master thesis I investigated the effects of 12 new benzodiazepines synthesized by the group of Jim Cook, University Wisconsin, USA, at $GABA_A$ receptors composed of αβγ subunits. The aim of this study was to determine a possible receptor subtype selectivity of these compounds as well as to investigate whether a structure-activity relationship could be established. For this, the *Xenopus laevis* oocytes were chosen as heterologous expression system. As they are benzodiazepines, these compounds should be able to bind at the BZ binding site. Given that the α+/γ- interface contains the same α+ side as the CGS binding site (α+/β-), it could be possible that some of these compounds can also bind at the α+β- site in αβ receptors. Therefore, it was investigated in addition whether these assumed BZ binding site ligands are active also at αβ receptors that do not contain a BZ binding site.

There is a big interest in creating subtype selective agonists because they may reduce the effects of physical dependence and the sedative effects but nevertheless, maintain the beneficial anticonvulsive and anxiolytic effects (Iversen, 2004).

2 MATERIALS AND METHODS

2.1 Chemicals

- Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one) (Sigma Aldrich, St. Louis, Missouri);
- Ro15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4] benzodiazepine-3-carboxylate) (Tocris Bioscience, Bristol, UK).

All 12 compounds used in this study were gifts of James Cook, University of Wisconsin-Milwaukee, Department of Chemistry and Biochemistry. Structures are given in Fig.9.
- CMS-I-26 (7-Bromo-5-(2-chloro-phenyl)-1,3,4,5-tetrahydro-benzo[*b*]azepin-2-one),
- CMS-I-45 (8-Bromo-6-(2-chloro-phenyl)-1-methyl-4*H*-2,3,5,10b-tetraaza-benzo[*e*]azulene),
- CMS-I-59 (6-(2-Chloro-phenyl)-1-methyl-8-trimethylsilanylethynyl-4*H*-2,3,5,10b-tetraazabenzo[*e*]azulene),
- JY-I-59 (4H-Imidazo[1,5-a][1,4]benzodiazepine-3-carboxylic acid, 8-ethynyl-6-(2-fluorophenyl)-, 2,2,2-trifluoroethyl ester),
- SH-I-030 ((*S*,*Z*)-7-bromo-3-methyl-5-phenyl-1*H*-benzo[*e*][1,4]diazepin-2(3*H*)-one),
- SH-I-044 ((*R*,*E*)-7-bromo-5-(2-fluorophenyl)-3-methyl-1*H*-benzo[*e*][1,4]diazepin-2(3*H*)-one),
- SH-I-049R ((*R*,*E*)-7-bromo-3-methyl-5-(pyridin-2-yl)-1*H*benzo[*e*][1,4]diazepin-2(3*H*)-one),
- SH-I-053B ((*R*,*Z*)-7-bromo-3-methyl-5-phenyl-1*H*benzo[*e*][1,4]diazepin-2(3*H*)one),
- SH-I-055 ((*S*,*E*)-ethyl 6-(2-fluorophenyl)-4-methyl-8-((trimethylsilyl)ethynyl)-4*H*benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxylate),
- SH-I-064 ((*R*,*E*)-ethyl 6-(2-fluorophenyl)-4-methyl-8-((trimethylsilyl)ethynyl)-4*H*benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxylate),
- SH-I-085 ((*E*)-5-(8-bromo-6-(2-fluorophenyl)-4*H*benzo[*f*]imidazo[1,5-*a*][1,4] diazepin-3-yl)oxazole),
- YT-III-19 (3,3'-(piperazine-1,4-dicarbonyl)bis(8-ethyl-5-methyl-4H-benzo[f] imidazo[1,5-a][1,4]diazepin-6(5H)-one)).

2.2. Receptor expression in *Xenopus leavis* oocytes

Oocytes of the *Xenopus laevis* frog were used for heterologous expression of $GABA_A$ receptors by injecting appropriate combinations of mRNAs of rat $GABA_A$ receptor subunits into the cells. Oocytes were isolated, cultured, injected and defolliculated as described previously (Sigel et al., 1990).

2.2.1 Oocyte preparation

Mature female *Xenopus laevis* (Nasco, Fort Atkinson, Wisconsin) were anaesthetized in a bath of ice-cold 0.17% tricain (Ethyl-m-aminobenzoat, Sigma Aldrich, St. Louis, Missouri), decapitated and then their ovary was removed. Positioned in a petri dish in Xenopus Ringer solution (XR solution, containing 90 mM NaCl, 5 mM HEPES-NaOH (pH 7.4), 1 mM $MgCl_2$, 1 mM KCl and 1 mM $CaCl_2$) under a stereo microscope, the mature oocytes at stage five to six with the follicle cell layer around were singled out of the ovary by using a platinum wire loop. For collection and transfer of the oocytes a Pasteur pipette with enlarged tip was used. The single oocytes were incubated at 18°C in modified Barths' Medium (88 mM NaCl, 10 mM HEPES-NaOH (pH 7.4), 2.4 mM $NaHCO_3$, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.34 mM $Ca(NO_3)_2$) with 100 U/ml penicillin and 100 µg/ml streptomycin added for about two hours to allow the oocytes to recover.

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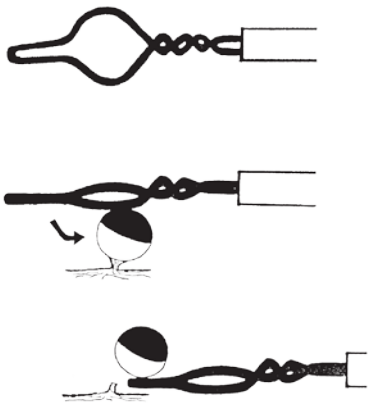


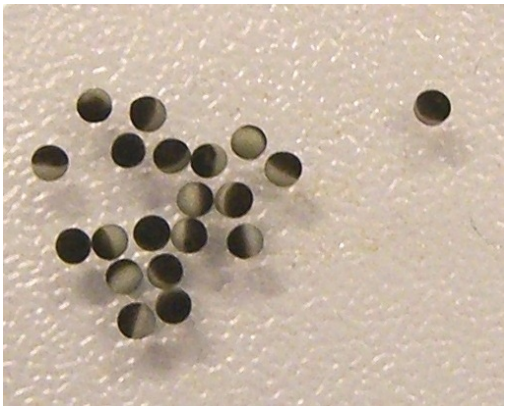


Fig.6: *Xenopus laevis* oocytes. A) Schematic representation of the mechanical isolation of single follicles from the ovary lobe by using a platinum wire loop (Sigel and Minier, 2005). B) Five to six stage singled-out oocytes. Living cells are firm in shape, have a stabile membrane with steady color and a clear border between the dark-brown (animal) and the white (vegetal) hemisphere. The animal hemisphere contains the cell's nucleus (self-made picture).

2.2.2 Injection of oocytes

After these two hours of recovering, the living oocytes, recognized by a firm shape and a sharp contrast between the two hemispheres, were placed on a nylon grid in a petri dish with XR solution and dead cells were discarded. The mixed recombinant mRNA solution from rats, containing the different subunit constructs in order to form the respective $GABA_A$ receptor, was stored in Eppendorf tubes at -80°C. mRNA constructs for α and β subunits were mixed in ratio 1:1, α, β and γ constructs in a 1:1:5 ratio. Each oocyte was injected with a total of 2.5 ng of mRNA (in 50 nl mRNA aliquots) encoding for different subtypes of $GABA_A$ receptors used in this study. Afterwards, oocytes were incubated for at least 24 hours at 18°C in Barth's medium supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin to allow the subunit-expression at the cell surface.

2.2.3 Defolliculation of oocytes

The procedure started by collagenase-treatment (~1 mg/mL, type IA, Sigma Aldrich, St. Louis, Missouri) containing 0.1 mg/mL trypsin inhibitor (type I-S from soybean C, Sigma Aldrich, St. Louis, Missouri) for an incubation time of 20 minutes at 37°C to destroy the connections between the oocyte and the follicular envelope. Secondly, the oocytes were washed in XR buffer. To facilitate the removal of the follicular cell layer, thirdly, cells were treated with a two times concentrated XR solution containing 3.8 mM EGTA (ethylene glycol tetra-acetic acid (Merck, Germany). Afterwards, oocytes were mechanically defolliculated by being rolled on an uncoated petri dish with a platinum wire loop and incubated in Xenopus Ringer solution containing antibiotic agents for another 24-48 hours (depending on the subunit composition) to give time for the expression and assembly of functional receptors. Defolliculated oocytes can be kept for up to 5 days provided that their incubation medium is changed every day and the dead cells are sorted out.

2.3 Two-electrode voltage clamp

After receptor expression at the surface membrane the influence of the compounds to be tested on GABA-induced currents could be measured using the two-electrode voltage clamp method.

2.3.1 Electrophysiological recordings

The measurements were performed at room temperature using a Warner OC-725C two-electrode voltage clamp (Warner Instrument, Hamden, CT). For electrophysiological measurements cells were placed on a nylon grid in a bath of XR buffer, and impaled with two microelectrodes filled with a 2 M KCl-solution and a resistance of 2-3 MΩ. Oocytes were clamped at a holding potential of -60 mV and the applied currents were registered by an amplifier together with the clamped potential. Cells were constantly washed by 6 ml/min of XR buffer. For measurements this buffer could be switched by the use of a two-way valve to an application of XR solution containing GABA and/or drugs.

A GABA concentration that triggers 3% of the respective maximum of GABA-elicited currents of the individual oocyte (EC_3) was titrated. Compounds were dissolved in DMSO and then adequately diluted into the XR solution containing either no GABA or GABA EC_3, resulting in a final concentration of 0.1% DMSO perfusing the oocytes. To test the modulatory effect of drugs on GABA-induced currents, different concentrations (1 nM to 10 µM) of the compound of interest in combination with GABA containing XR solution (EC_3) were applied to the cell for about 50 seconds until a peak response was observed. To ensure receptor recovery from desensitization, cells were washed with XR buffer for 3 to 15 minutes depending on the applied GABA concentration after each measurement. Between the applications of the various compounds, the perfusion system was washed with 100% DMSO.

To investigate whether the cell was in a stable equilibrium condition, each cell was tested by applying two consecutive pulses of GABA at EC_3.The correct assembly of receptors containing a γ2 subunit was investigated by stimulating GABA-induced currents by 1 µM diazepam and afterwards comparing the extent of stimulation with that of reference data. For inhibition experiments the high affinity null modulator Ro 15-1788 was used at a concentration of 10 µM. At the end of each measurement a 1 mM GABA solution, representing a receptor-saturating concentration, was applied.

Since receptor expression varies from cell to cell leading to different peak-currents at the same GABA concentrations, it was necessary to normalize. The GABA EC_3 current was normalized to 100% and the GABA modulatory effect of the compound in respect to the normalized GABA EC_3 was calculated. Any effect higher or lower than 100% showed a GABA modulatory effect of the compound.

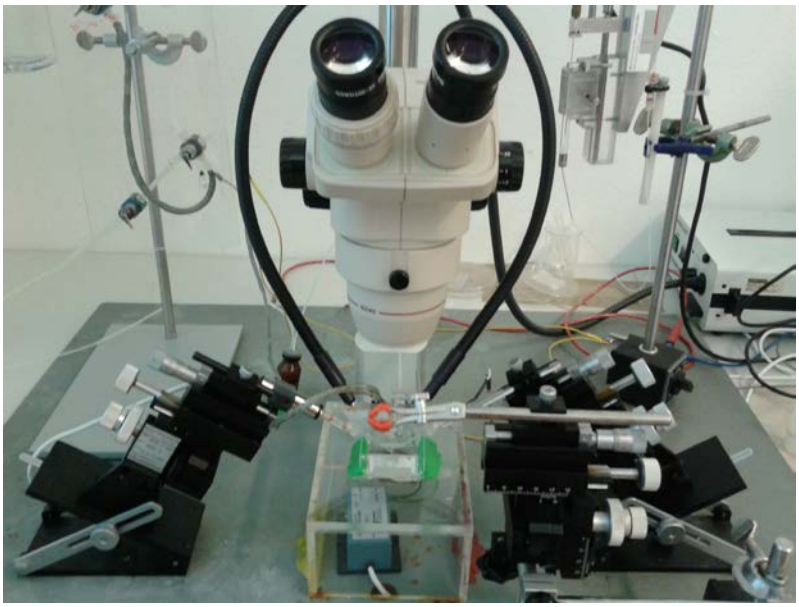


Fig.7: The picture shows our two-electrode voltage clamp setup. Cells are placed in a bath (between green mark) containing Xenopus Ringer solution below a stereomicroscope and get impaled with two measurement electrodes by using micromanipulators (black, on each side). Right on top of the cell is the applicator (orange) that is connected with a liquid suction system (self-made picture).

2.3.2 Analysis

Electrophysiological data were digitized, recorded and measured using a Digidata 1322A data acquisition system (Axon Instruments, Union City, CA). For data recording the programs Clampfit and Clampex (Axon Instruments, Union City, CA), for data analysis GraphPad Prism program (GraphPad Software, CA, USA) were used.

Data represent means ± SEM from at least two oocytes derived from more than one oocyte batch. When only two measurements were performed the arithmetic mean of the two values was given.

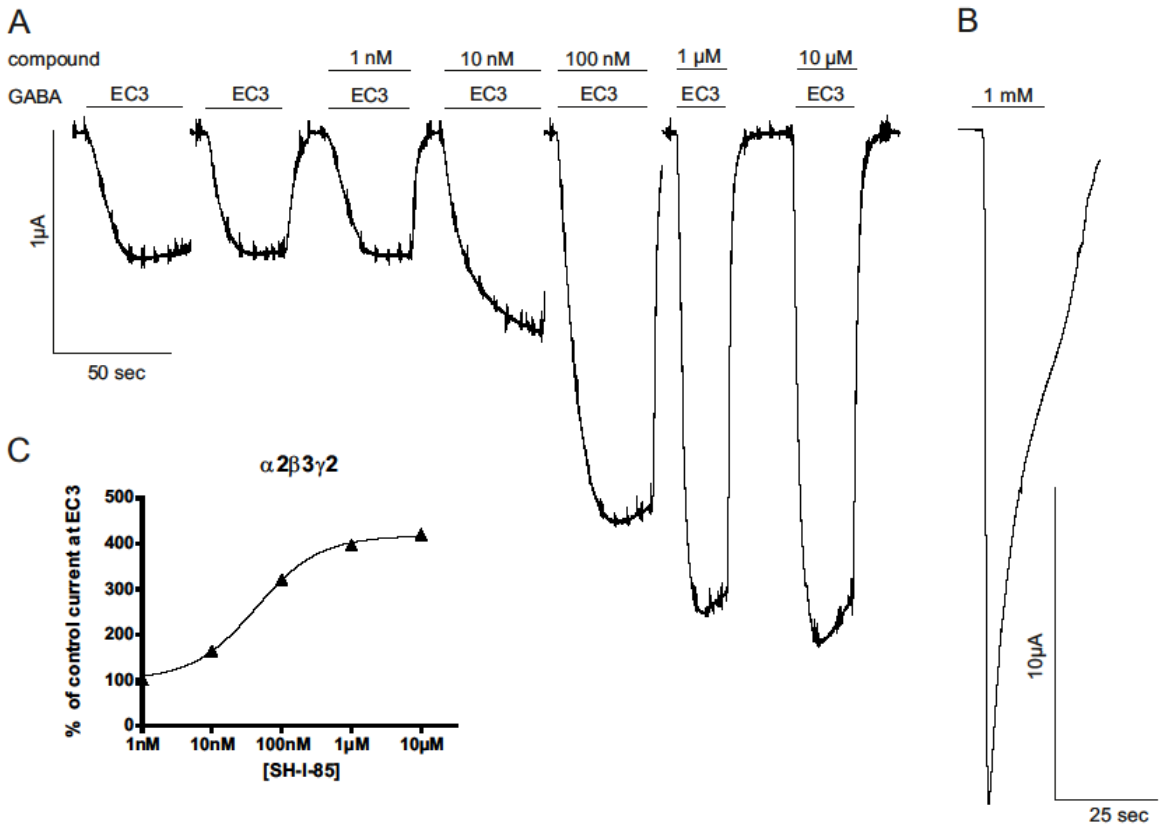


Fig.8: Example for data analysis representing the dose-dependent effects of compound SH-I-85 at an oocyte expressing α2β3γ2 $GABA_A$ receptors. A) Raw data traces for compound SH-I-85 at increasing concentrations (1-10 000 nM) in the presence of GABA at EC_3 (the concentration of GABA, which triggers 3% of the maximum GABA effect) B) Current trace evoked by 1 mM GABA applied at the beginning and at the end of the measurement to determine EC_3 and the drift in measurements in the oocytes. C) Dose response curve created with GraphPad Prism program to show compound-concentration dependent effect at EC_3. Figure created with CorelDRAW X6.

3 RESULTS

I tested 12 different compounds which belong to the group of benzodiazepines on their ability to modulate GABA-induced currents at $GABA_A$ receptors. GABA currents were measured in the presence of various concentrations of these compounds at different receptor compositions. I used α1, 2, 3, 5 subunits in combination with β3 and γ2 subunits and α1, 2, 3, 5 with β3 subunits, only. None of the compounds showed effects in the absence of GABA.

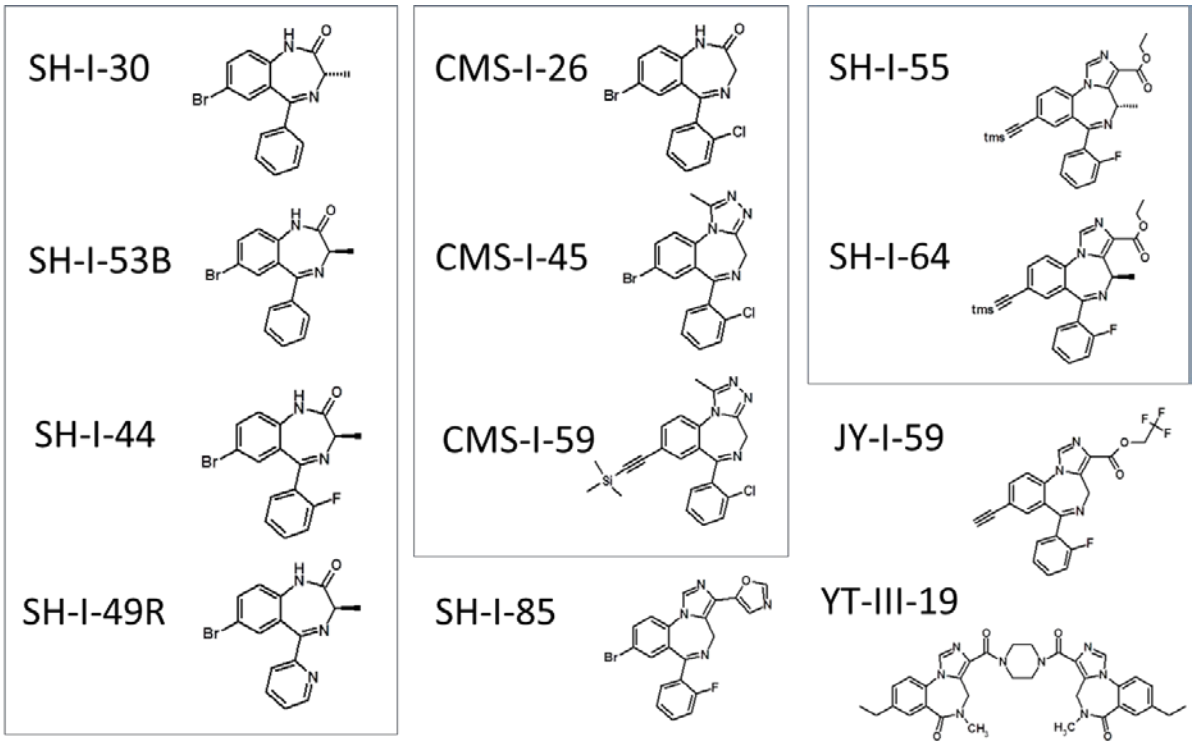


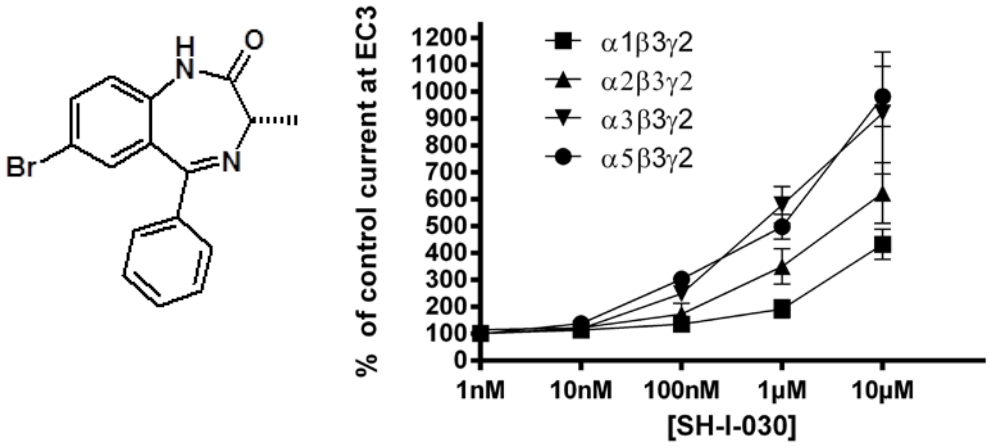
Fig.9: Structures of compounds used in this study, arranged according to similarity of their chemical structure.

3.1 Classical benzodiazepines exhibit different effects depending on the orientation of the methyl group at position 3 of the diazepine ring

SH-I-30 versus SH-I-53B

The methyl group at position three of the diazepine ring has a different orientation in compounds SH-I-30 and SH-I-53B.

A



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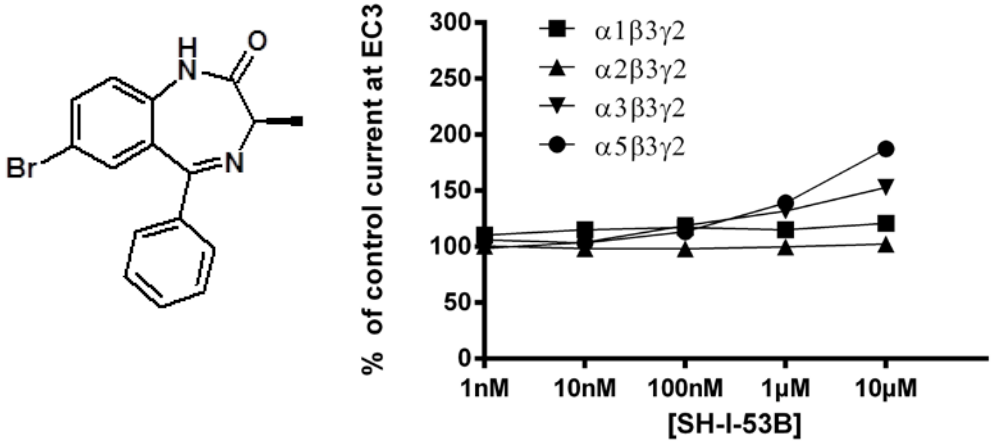


Fig.10: Modulation of GABA EC_3 by various concentrations of A) SH-I-30 (n=3-4) and B) SH-I-53B (n=2) performed at α1, α2, α3, α5 β3γ2 receptors. Data are normalized to control current (100%) at GABA EC_3. Data points represent means ± SEM from at least 2 oocytes derived from >1 cell batches. When only 2 measurements were performed the arithmetic mean of the two values is given.

The compound SH-I-30 enhanced GABA-induced currents at all receptor subtypes investigated, but the effects observed were higher at $\alpha3\beta3\gamma2$ and $\alpha5\beta3\gamma2$ receptors than at $\alpha1\beta3\gamma2$ or $\alpha2\beta3\gamma2$ receptors. Thus, this compound was able to stimulate the effects of $\alpha3\beta3\gamma2$ and $\alpha5\beta3\gamma2$ receptors significantly up to 300% of GABA EC_3 already at 100 nM concentrations ($\alpha3$: 249 ± 26, $\alpha5$: 304 ± 21%), whereas stimulation at this concentration at $\alpha1\beta3\gamma2$ and $\alpha2\beta3\gamma2$ receptors was quite weak ($\alpha1$: 136 ± 16%, $\alpha2$: 174 ± 39%). At this concentration this compound thus showed some $\alpha3\beta3\gamma2$ and $\alpha5\beta3\gamma2$ receptor subtype preference.

At 1 μM or 10 μM concentrations, GABA-induced currents at $\alpha1\beta3\gamma2$ and $\alpha2\beta3\gamma2$ receptors were also enhanced, but not as much as at $\alpha3\beta3\gamma2$ and $\alpha5\beta3\gamma2$ receptors. Overall, at 10 μM concentrations this compound exhibited the strongest stimulation of GABA-induced currents of all compounds investigated. Equally strong effects were measured for $\alpha3\beta3\gamma2$ (922 ± 227%) and $\alpha5\beta3\gamma2$ (983 ± 112%) at 10 μM, followed by a weaker potentiation of $\alpha2\beta3\gamma2$ containing receptors (624 ± 112%) and the relatively weakest effect at $\alpha1\beta3\gamma2$ receptors (433 ± 56%).

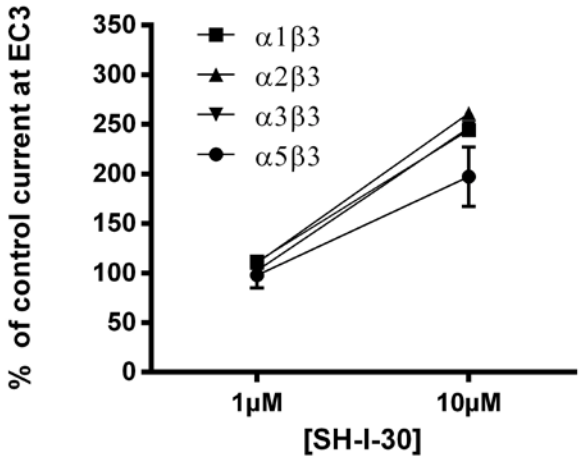
In contrast, the compound SH-I-53B which is a stereoisomer of SH-I-30 did not enhance GABA-induced currents in the nM range (see Table 3 (page 41), stimulation <120% was not significant) and did not show potentiation stronger than 200% of GABA EC_3 at any receptor combination at 10 μM concentrations. But as with SH-I-30, the receptors containing $\alpha3$ or $\alpha5$ subunits showed the highest effects with compound SH-I-53B.

Interestingly, the effects of SH-I-30 at 10 μM concentrations were still not saturated, although saturation would have been expected to occur within two orders of magnitude of drug concentrations if only one binding site was used by this compound. It was thus possible that more than one binding site contributed to the effects of SH-I-30. We therefore investigated whether this compound might interact also with receptors containing only α and β subunits. Recently it was demonstrated, that other benzodiazepine binding site ligands (CGS 9895 and other pyrazoloquinolinones, (Ramerstorfer et al., 2011; Varagic et al., 2013a; Varagic et al., 2013b) not only interact via the benzodiazepine binding site but also with a second binding site located at the extracellular $\alpha+\beta$- interface of $GABA_A$ receptors.

3.2 Investigation of compounds at αβ receptors

In order to investigate a possible interaction with a second binding site, the effects of all compounds on αβ receptors were investigated. To reduce the experimental effort for this screening, compounds SH-I-30 (Fig.11), SH-I-85 (not shown) and JY-I-59 (not shown) were investigated at αβ receptors with n=2-3 and the remaining 9 compounds mostly with n=1 in α1β3, α2β3, α3β3, or α5β3 receptors.

A



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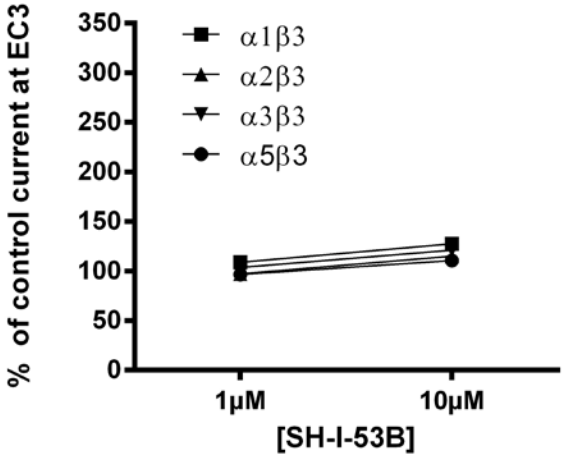


Fig.11: Modulation of GABA EC_3 by A) SH-I-30 (n=2-3) and B) SH-I-53B (n=1-2) at α1, 2, 3, 5 β3 receptors.

As shown in Fig.11, compound SH-I-30 enhanced GABA-induced currents at αβ receptors at 10 μM but not at 1 μM concentrations. Although the effects were much lower than at αβγ receptors, SH-I-30 potentiated GABA EC_3 currents up to 244% at α1β3, 261% at α2β3, 247% at α3β3 and 197 ± 30% at α5β3 receptors at concentrations of 10 μM. In contrast, such a stimulatory effect was not seen with the structurally similar compound SH-I-53B, which exhibited nearly no effect (<130%) at αβ receptors. From all compounds tested only SH-I-30 exhibited effects over 200% at αβ receptors at 10 μM concentrations, the other 11 compounds could barely enhance GABA-induced currents at receptors without the γ subunit incorporated.

3.3 Inhibition of compound effects by Ro 15-1788

Ro 15-1788 (flumazenil) is a high affinity antagonist (high affinity null modulator) at the benzodiazepine binding site. It binds at the α+/γ- interface of the $GABA_A$ receptor and competitively inhibits the effects of benzodiazepines. SH-I-30 seems to modulate GABA-induced currents in αxβ3γ2 and αxβ3 receptors. In order to prove that the effects of SH-I-30 at αβγ receptors are mediated via the benzodiazepine binding site, inhibition experiments were performed comparing the effects of the compound with its effects in combination with Ro 15-1788 in a 1:10 ratio. The antagonist Ro 15-1788 should inhibit the effects of SH-I-30 if they both act via the same binding site.

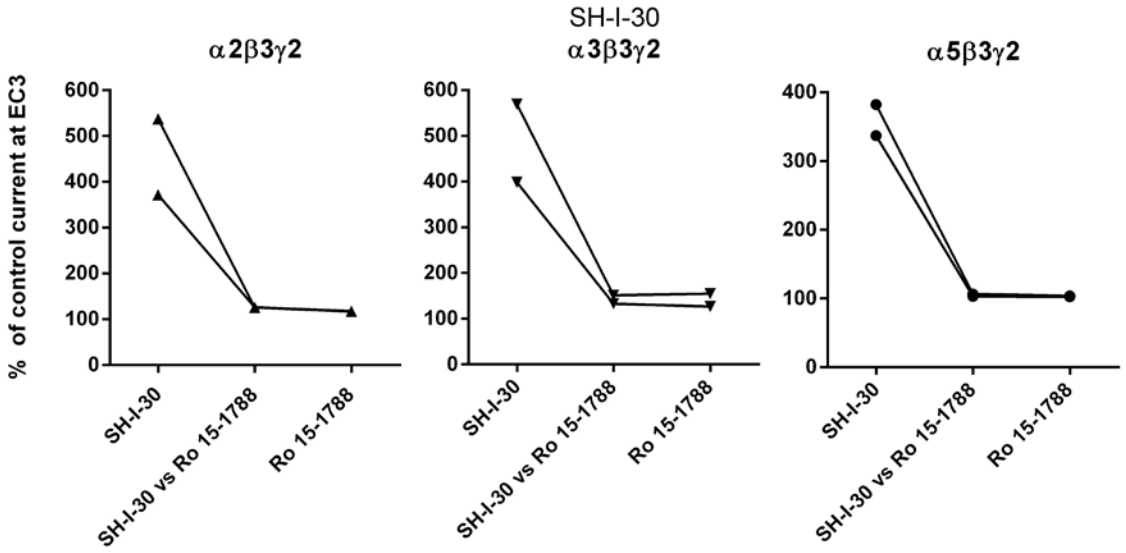


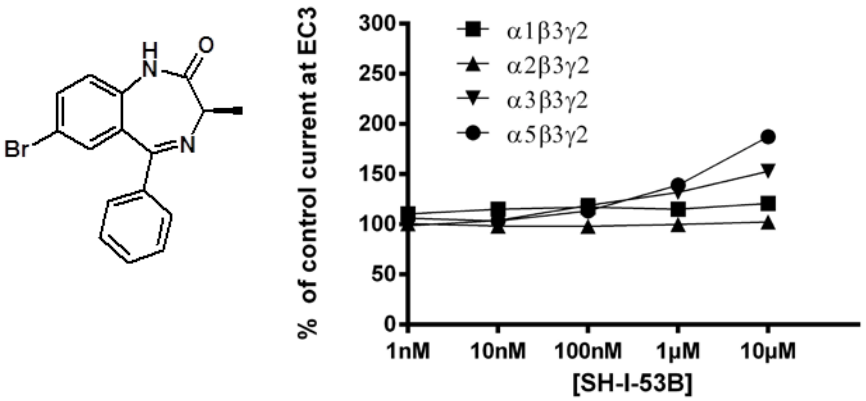
Fig.12: Effects of 1 µM SH-I-30 in the absence or presence of 10 µM Ro 15-1788 at α2, α3 and α5β3g2 receptors. Data represent two independent measurements performed in two oocytes and are normalized to a control GABA current at EC_3.

SH-I-30 at a concentration of 1 µM was able to stimulate GABA EC_3 to 340%-570% depending on the α subunit in αxβ3γ2 receptors. The weak effect of this compound at α1β3γ2 receptors was not investigated. After addition of 10 µM Ro 15-1788 there was a clear reduction of the effects. At α2β3γ2 receptors 98% and at α5β3γ2 receptors 99% of the effect of SH-I-30 could be inhibited by Ro 15-1788, at α3 containing receptors Ro 15-1788 reduced the compound's effect to 142% of GABA EC3, a value comparable with the effects elicited by Ro 15-1788 alone. In previous studies (Ramerstorfer et al., 2010) it has been demonstrated that Ro 15-1788 was able to weakly potentiate GABA-induces currents in α3β3γ2 receptors. Thus, the effects of Ro 15-1788 alone of about 150% at α3β3γ2 receptors measured in the present experiments were as expected. As the effects of SH-I-30 could be more or less completely inhibited by Ro 15-1788, we can assume that the compound at 1 µM concentration indeed exhibits its effects via the BZ binding site at α2, 3, 5 β3γ2 receptors.

3.4 Changes in the pendant phenyl ring influence efficacy

SH-I-53B versus SH-I-44 versus SH-I-49R

A



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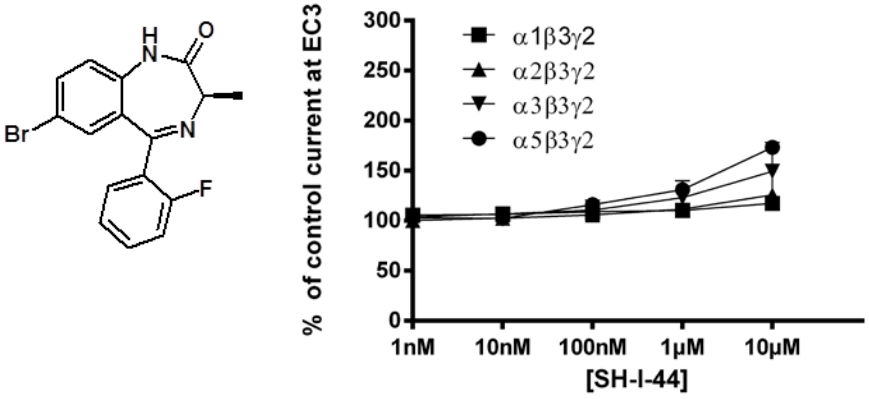

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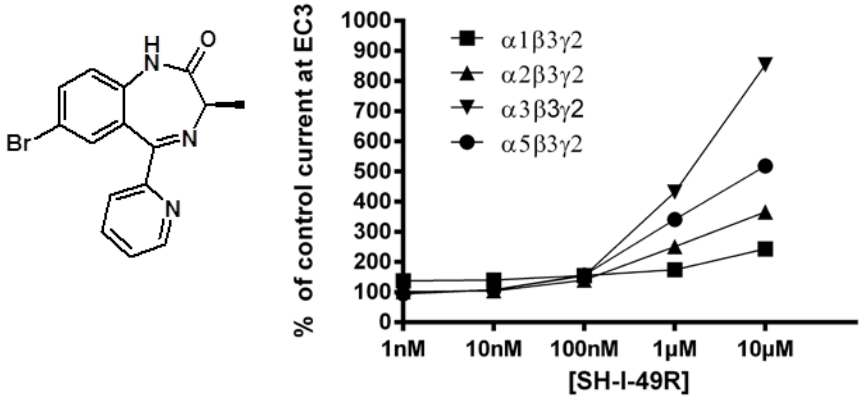


Fig.13: Molecular structure and concentration-dependent modulation of GABA EC_3 by A) SH-I-53B (n=2), B) SH-I-44 (n=2-3), and C) SH-I-49R (n=2), at αxβ3γ2 receptors. Data are normalized to control currents at GABA EC_3. Data points represent means ± SEM from at least 2 oocytes derived from >1 cell batches. When only 2 measurements were performed the arithmetic mean of the two values is given.

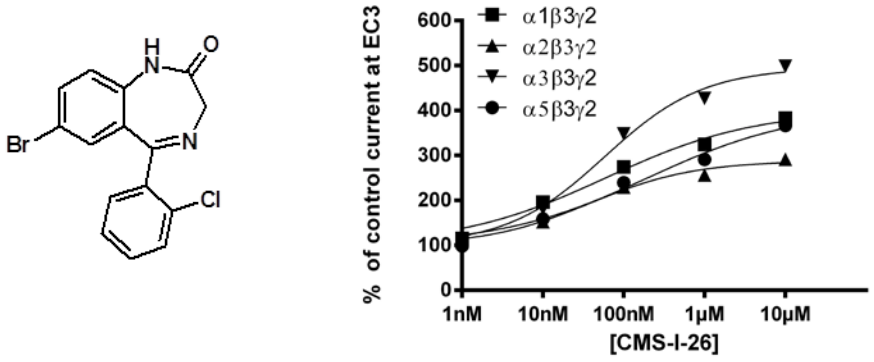
Compound SH-I-44 differs from SH-I-53B by a substitution with ortho-fluorine at the pendant phenyl ring, but nevertheless behaves in a similar way. The highest effects were achieved at $\alpha 5$ containing receptors, followed by $\alpha 3\beta 3\gamma 2$ receptors. None of these two compounds significantly enhanced GABA-induced currents in the nM range (<120%) and they both show very low effects in the µM range (<190%).

This situation differed when a pyridine ring with the nitrogen in ortho position replaced the pendant phenyl ring as in compound SH-I-49R. This compound exhibited an enhanced efficacy at all receptor combinations measured. At 10 µM concentrations GABA-induced current was enhanced to about 244% at $\alpha 1\beta 3\gamma 2$, 366% at $\alpha 2\beta 3\gamma 2$, 519% at $\alpha 5\beta 3\gamma 2$ and up to 855% at $\alpha 3\beta 3\gamma 2$ receptors. Nevertheless this compound exhibited a low potency at all receptors investigated (between 140% and 160% potentiation of GABA EC_3 at a concentration of 100 nM). In contrast to SH-I-44 and SH-I-53B which exhibited their highest effects at receptors containing the $\alpha 5$ subunits, compound SH-I-49R exhibited its highest efficacy at the $\alpha 3$ containing receptor (855% at 10 µM concentrations).

3.5 Triazolobenzodiazepines and classical benzodiazepines behave similarly

CMS-I-26 versus CMS-I-45 versus CMS-I-59

A



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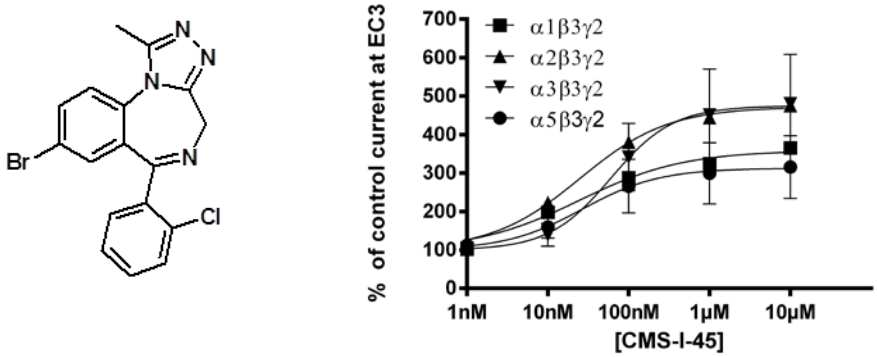

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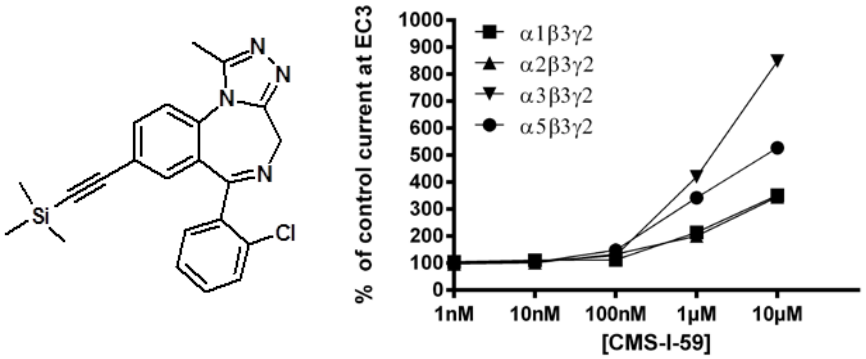


Fig.14: Molecular structure and concentration-dependent modulation of GABA EC_3 by A) CMS-I-26 (n=2) B) CMS-I-45 (n=2-3) and C) CMS-I-59 (n=2) at αxβ3γ2 receptors. Data are normalized to control GABA currents at EC_3. Data points represent means ± SEM from at least 2 oocytes. When only 2 measurements were performed the arithmetic mean of the two values is given.

Compound CMS-I-45 is an analogue of triazolam (Halcion; 8-chloro-6-(2-chlorophenyl)-1-methyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine), in which the chloro group at position 8 is replaced by a bromo group.

A comparison between the compounds CMS-I-26 and CMS-I-45 allowed the investigation of the effects of the triazolo ring at GABA EC_3. Both compounds showed interesting potency in the nM range, but the effects were rather similar in each receptor combination. CMS-I-26 showed the highest stimulation of 499 ± 35% at $\alpha3\beta3\gamma2$ receptors at 10 µM, whereas $\alpha1$ and $\alpha5$ containing receptors showed a stimulation of 383% and 367%, $\alpha2\beta3\gamma2$ receptors were only stimulated to 292% (Table 3, page 41). Although there were effects in the 100 nM range between 230-350% (275%, 230%, 348%, 240% for the individual receptor subtypes, respectively), there was no preference for one specific receptor subtype. CMS-I-45 acted in almost the same manner, it showed a high potency but no clear subtype selectivity in the nM range. The only difference between these two compounds was that $\alpha2$ containing receptors showed much higher effects with CMS-I-45 (381% at 100 nM) than with CMS-I-26 (230% at 100 nM).

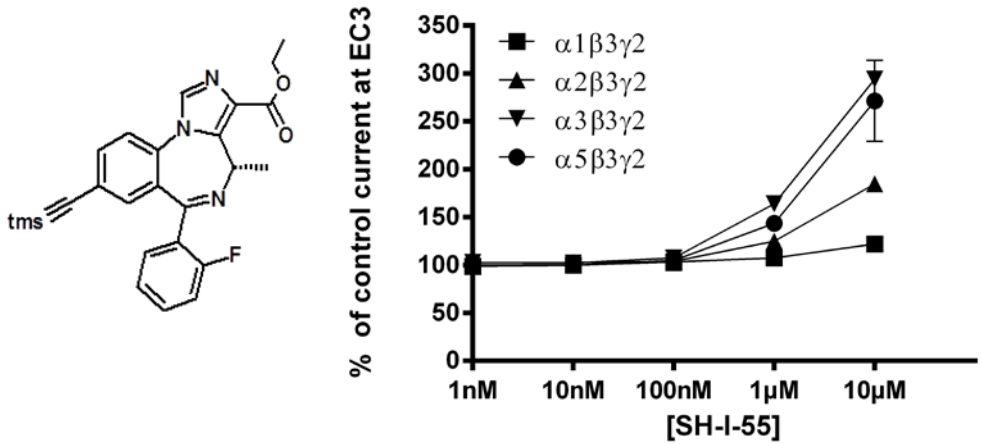
In compound CMS-I-59 the bromo-group is replaced by a much bigger acetylene-trimethyl-silyl-group. This led to a reduced potency. The effects in the nM range were <150% in all receptor subtypes. Stimulation of GABA EC_3 in the µM range was higher for $\alpha3$ and $\alpha5$ subunit containing receptors, than for $\alpha1$ and $\alpha2$ subunit containing receptors (Table 3).

3.6 Orientation of the methyl group in imidazobenzo-diazepines influences the efficacy of $\alpha3\beta3\gamma2$ receptors

SH-I-55 versus SH-I-64

The compounds SH-I-55 and SH-I-64 only differ in the direction of the methyl group at position three of the diazepine ring.

A



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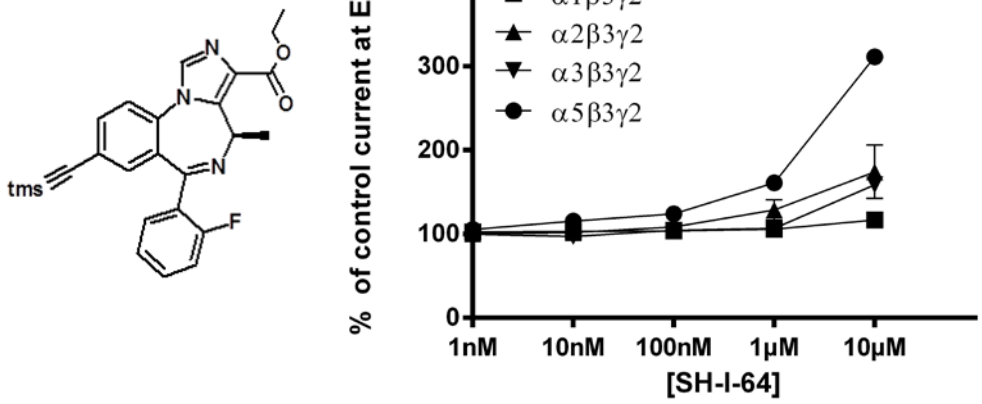

Fig.15: Molecular structure and concentration-dependent modulation of GABA EC_3 by A) SH-I-55 (n=2-3) and B) SH-I-64 (n=2-3) at αxβ3γ2 receptors. Data are normalized to control GABA currents at EC_3. Data points represent means ± SEM from at least 2 oocytes. When only 2 measurements were performed the arithmetic mean of the two values is given.

The compound SH-I-55 exhibited low potency because in all measured receptor combinations there was no effect (<110%) in the nM range. At the highest concentration of 10 µM the compound caused a stimulation of GABA-induced currents of 185% at α2β3γ2, 294% at α3β3γ2, 271 ± 42% at α5β3γ2 and nearly no stimulation at α1β3γ2 receptors (122%). By changing the orientation of the methyl group the effects at receptors containing the α3 subunit decreased to 159% at 10 µM. In all other receptor subunit combinations SH-I-64 exhibited nearly the same effects as SH-I-55 (Table 3).

3.7 Compounds exhibiting α subtype selectivity

In addition to compound SH-I-30, that was discussed in chapter 3.1 at page 28-29, and that exhibited some selectivity for α3 and α5 receptors at 100 nM concentrations, other compounds in this series also exhibited a certain α subtype selectivity.

SH-I-85

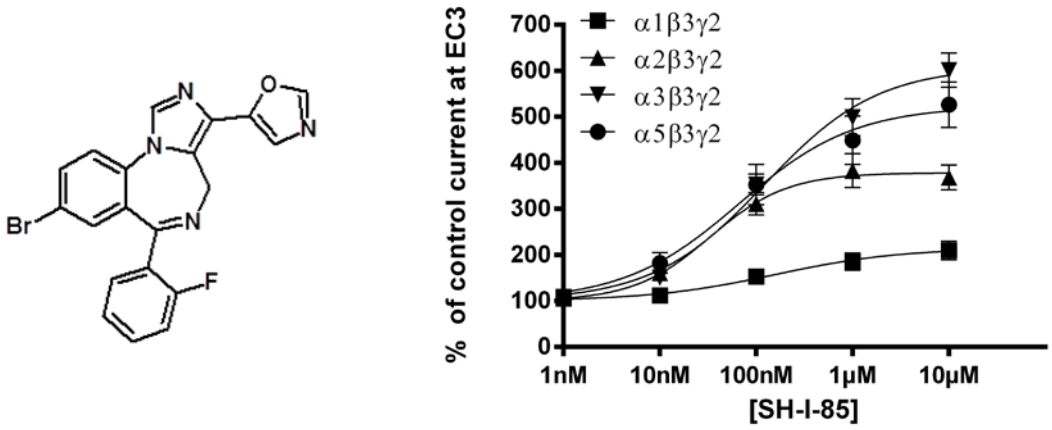

Fig.16: Molecular structure and concentration-dependent modulation of GABA EC_3 by SH-I-85 (n=3) at α1, α2, α3, α5 β3γ2 receptors at GABA EC_3. Data are normalized to control currents at GABA EC_3. Data points represent means ± SEM from at least 3 oocytes derived from >1 cell batches.

The compound SH-I-85 showed different efficacy at the highest concentration of 10 µM. At α3β3γ2 and α5β3γ2 receptors the compound stimulates GABA EC_3 significantly up to 600% (α3: 601 ± 37%, α5: 526 ± 49%), whereas stimulation at this concentration at α1β3γ2 (209 ± 20%) and α2β3γ2 (368 ± 27) receptors was weaker. SH-I-85 has a high potency for α2, 3, 5 containing receptors because it already enhanced GABA-induced currents up to 350% at a concentration of 100 nM (α2: 311 ± 24%, α3: 353 ± 23%, α5: 352 ± 44%). In contrast, at α1 containing receptors a far weaker effect (153 ± 12%) was exhibited at this concentration. This results in a selective effect at 100 nM as the compound showed a different stimulating potential depending on the receptor composition.

Table 1: Potency of SH-I-85

Compound	Receptor	EC_{50} (nM)
SH-I-85	$\alpha1\beta3\gamma2$	158
	$\alpha2\beta3\gamma2$	33
	$\alpha3\beta3\gamma2$	124
	$\alpha5\beta3\gamma2$	67

Since the effects of this compound were saturated at all receptor combinations investigated, EC_{50} values could be calculated (158 nM for $\alpha1\beta3\gamma2$, 33 nM for $\alpha2\beta3\gamma2$, 124 nM for $\alpha3\beta3\gamma2$ and 67 nM for $\alpha5\beta3\gamma2$ receptors).

JY-I-59

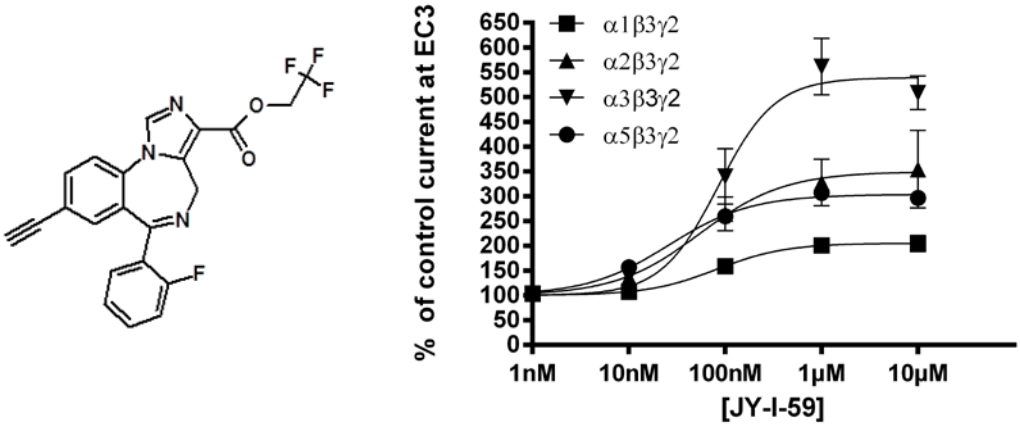

Fig.17: Molecular structure and concentration-dependent modulation of GABA EC_3 by JY-I-59 (n=3) at $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$ $\beta3\gamma2$ receptors in the presence of GABA at EC_3. Effects are normalized to control currents at GABA EC_3. Data points represent means ± SEM from 3 oocytes derived from >1 cell batches.

The compound JY-I-59 enhanced GABA-induced currents at all receptor subtypes investigated, but a strong preference for $\alpha3$ containing receptors (509 ± 34% at 10 µM) could be observed. In contrast, the effects for $\alpha2$ (355 ± 78%), $\alpha5$ (296 ± 11%) and especially $\alpha1$ (205 ± 16%) subunit containing receptors were far weaker at 10 µM. At this high concentration the compound thus suggests an $\alpha3$ subtype selectivity. At 100 nM, $\alpha3$ containing receptors showed effects of 340 ± 56% followed by equally strong effects measured for $\alpha2$ and $\alpha5$

containing receptors (264 ± 34%, 260 ± 12%) whereas stimulation at this concentration at $\alpha1\beta3\gamma2$ receptors was quite weak (159 ± 3%). At all receptor combinations saturation is taking place at concentrations of 1µM.

Table 2: Potency of JY-I-59

Compound	Receptor	EC_{50} (nM)
JY-I-59	$\alpha1\beta3\gamma2$	82
	$\alpha2\beta3\gamma2$	55
	$\alpha3\beta3\gamma2$	85
	$\alpha5\beta3\gamma2$	26

YT-III-19

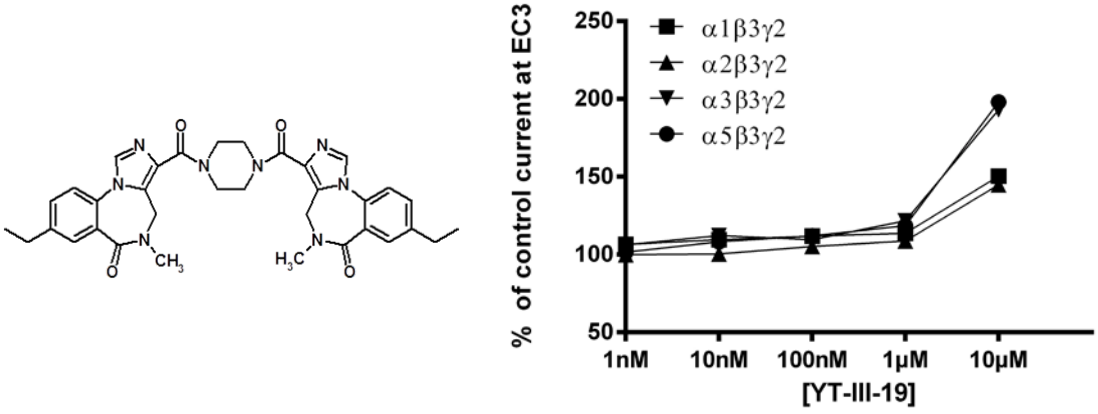

Fig.18: Molecular structure and concentration-dependent modulation of GABA EC_3 by YT-III-19 (n=2) at $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$ $\beta3\gamma2$ receptors in the presence of GABA at EC_3. Data are from two different oocytes and represent the arithmetic mean of the two values.

The compound YT-III-19 is structurally totally different from the other 11 compounds and cannot directly be compared with the others. It showed a very low potency with little effects in the nM range (<115%) and even at a concentration of 1 µM the compound was not able to stimulate GABA EC_3 to more than 125%. At these low concentrations all receptor combination showed similar effects. In contrast, at 10 µM $\alpha1$ and $\alpha2$ containing receptors were stimulated to 151% and 145%, respectively, whereas $\alpha3$ and $\alpha5\beta3\gamma2$ receptors show a similarly increased stimulation to 193% and 198% of the control current at EC_3, respectively. At this concentration the compound points towards an $\alpha3/\alpha5$ subtype selectivity.

Table 3: Effects of all 12 compounds at αβγ receptors. Values are given as means ± SEM (n≥3) and if n=2 only means are shown.

compound	concentration	α1β3γ2	α2β3γ2	α3β3γ2	α5β3γ2
SH-I-30	100 nM	136 ± 16	174 ± 39	249 ± 26	304 ± 21
n≥3	1 µM	192 ± 33	350 ± 66	579 ± 68	498 ± 47
	10 µM	433 ± 56	624 ± 112	922 ± 227	983 ± 112
SH-I-53B	100 nM	117	98	119	114
n≥2	1 µM	115	100	132	139
	10 µM	121	102	153	187
SH-I-44	100 nM	109 ± 2	106	111 ± 10	116
n≥2	1 µM	110 ± 1	112	123 ± 17	131
	10 µM	117 ± 3	126	149 ± 29	173
SH-I-49R	100 nM	156	140	155	157
n≥2	1 µM	174	251	432	341
	10 µM	244	366	855	519
CMS-I-26	100 nM	275	230	348	240
n≥2	1 µM	325	257	427	291
	10 µM	383	292	499	367
CMS-I-45	100 nM	288	381	342 ± 88	266 ± 70
n≥2	1 µM	325	444	451 ± 119	299 ± 80
	10 µM	365	476	480 ± 129	316 ± 81
CMS-I-59	100 nM	113	134	129	149
n≥2	1 µM	215	201	420	343
	10 µM	352	346	848	528
SH-I-55	100 nM	104	104	108	105 ± 10
n≥2	1 µM	107	125	164	144 ± 7
	10 µM	122	185	294	271 ± 42
SH-I-64	100 nM	104	108 ± 6	105	124
n≥2	1 µM	106	129 ± 12	107	161
	10 µM	117	174 ± 32	159	312
SH-I-85	100 nM	153 ± 12	311 ± 24	353 ± 23	352 ± 44
n≥3	1 µM	185 ± 19	383 ± 37	499 ± 40	449 ± 52
	10 µM	209 ± 20	368 ± 27	601 ± 37	526 ± 49
JY-I-59	100 nM	159 ± 3	264 ± 34	340 ± 56	260 ± 12
n≥3	1 µM	201 ± 11	328 ± 47	562 ± 57	307 ± 14
	10 µM	205 ± 16	355 ± 78	509 ± 34	296 ± 11
YT-III-19	100 nM	112	105	110	112
n≥2	1 µM	114	109	122	119
	10 µM	151	145	193	198

4 DISCUSSION

The 12 novel benzodiazepines investigated in this study have been synthesized by the group of James Cook, University Wisconsin-Milwaukee, with the aim to generate compounds as potent as diazepam but with receptor subtype selective actions.

In the present study it was investigated how these compounds affect GABA-induced currents at $\alpha x\beta 3\gamma 2$ $GABA_A$ receptors containing $\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 5$ subunits by using electrophysiological studies in *Xenopus laevis* oocytes as heterologous expression system for various receptor subtypes. Receptors containing $\alpha 4$ or $\alpha 6$ subunits were not investigated because previous results have demonstrated that such receptors are not modulated by compounds of this structural class. Since this was a screening study, the experiments in most cases were performed in duplicates, and only if a compound seemed to be interesting, they were performed in triplicates. Overall, these measurements clearly identified structural features that enhance or reduce the potency and efficacy at the benzodiazepine binding site of $GABA_A$ receptors.

The new benzodiazepines showed some interesting properties. As typical for allosteric modulators like benzodiazepines, none of the compounds was able to directly open the $GABA_A$ receptor-intrinsic chloride channel at the highest concentration of 10 µM applied in the absence of GABA. The compounds, however, were able to modulate GABA-induced currents at at least some $GABA_A$ receptor subtypes, but in most cases exhibited only low effects in the nM range. For a possible clinical application compound effects in the nM range are important, because *in vivo* usually only a maximum concentration around 100 nM can be reached by usually applied doses of benzodiazepines. So to possibly achieve clinical importance an effect at this concentration should be observed. Moreover, the data indicated that the majority of compounds showed similar effects at all subunit combinations investigated. Only four compounds exhibited some receptor subtype selectivity, being able to differentially stimulate GABA-induced currents in receptors containing different α subunits. Three of these compounds also exhibited their effects already at nM concentrations and thus, in the future will be investigated in behavioral experiments in rats by a laboratory collaborating with us. If interesting

properties are detected in these experiments, these compounds might enter a possible clinical development.

4.1 The chemical structure of benzodiazepines influences the effects at αβγ $GABA_A$ receptors

The knowledge on how the chemical structure influences the efficacy and the potency of a compound at $GABA_A$ receptors exhibiting a specific subunit composition can be used for further synthesis of improved subtype selective ligands. In this study at least some correlations between the efficacy and the molecular structure of compounds could be identified.

Only a few compounds investigated exhibited GABA-enhancing effects already at nM concentrations. One of these compounds was SH-I-30 (p28), that elicits an α3 and α5 receptor selective stimulation to about 250-300% of GABA EC_3 already at 100 nM concentrations. CMS-I-26 and CMS-I-45 were able to elicit comparable effects (stimulation to 250-350% of GABA EC_3) at 100 nM concentration at all receptor subtypes investigated (p35). Compounds SH-I-85 (p38) and JY-I-59 (p39) exhibited a selectivity for α2, α3, and α5 receptors and also stimulated these receptors at 100 nM concentration to 250-350% of GABA EC_3. Changes in the substitution of these compounds, however, resulted in a reduced potency.

For instance, **SH-I-30** and **SH-I-53B** differ only in the direction of the methyl group at position three of the diazepine ring, but the two compounds showed huge differences in efficacy (p28). SH-I-30 not only was much more potent in modulating GABA-induced currents, it also exhibited α3 and α5 receptor selectivity at 100 nM concentrations and at higher concentrations exhibited the highest effects of all compounds investigated. In contrast, SH-I-53B only marginally enhanced GABA-induced currents at all αβγ receptors, and the effects were observed only at μM concentrations. A different orientation of the 3-methyl group thus drastically reduces the potency and efficacy of these classical benzodiazepines.

There is a different situation in the other stereoisomer couple of this study: The imidazobenzodiazepine stereoisomers **SH-I-55** and **SH-I-64** again differ only

by the orientation of the same methyl group, but exhibited nearly the same effects at a concentration of 10 μM at all $GABA_A$ receptor subunit combinations (p37). Only at receptors containing the α3 subunit the efficacy was decreased after changing the orientation of the methyl group. Thus, the methyl group orientation in the case of the imidazobenzodiazepines influences the effects of compounds in the same direction as with the classical benzodiazepines, but only at α3β3γ2 receptors. The additional differences in the structure of the compounds (7-Br versus the large 8–(trimethylsilyl)ethynyl- group; classical benzodiazepine versus substituted imid-azobenzodiazepine) might have contributed to these diverging effects.

Although the compounds **SH-I-44** and **SH-I-53B** differ by a substitution with ortho-fluorine at the pendant phenyl ring, they behave in a similar way (p33). From that it can be concluded that this substitution has hardly any influence on the activity of the compound. On the contrary, when the pendant phenyl ring of SH-I-53B was replaced by a pyridine ring with the nitrogen in ortho position as with compound **SH-I-49R**, an enhanced efficacy could be observed. Thus, on the one hand the pyridine ring was responsible for an increased efficacy and on the other hand the highest effects were seen at α3 containing receptors instead of a highest effect at receptors containing the α5 subunits as seen with SH-I-44 and SH-I-53B. Given the above conclusion that the other orientation of the 3-methyl group would result in compounds with higher potency and efficacy, it would be interesting to investigate the effects of the compound SH-I-30 in which the pendant phenylring is replaced by a pyridine ring with the nitrogen in ortho position as in compound SH-I-49R (presumed name: compound SH-I-49S). Currently, this compound is not available to us.

Compound **CMS-I-26** (p35) differs from **SH-I-30** (p28) by lacking the 3-methyl group and by the additional presence of an ortho-Cl at the pendant phenyl ring. Both compounds exhibit a high potency and a comparable modulation of GABA-induced currents at 100 nM concentration, but differ in the relative activation of different receptor subtypes. At this concentration especially the modulation of α1β3γ2 receptors by CMS-I-26 is much higher and that of α5β3γ2 receptors is lower than by SH-I-030. Effects at 10 μM concentration of SH-I-030 are much higher than those of CMS-I-26, but are mediated via interaction of this compound with a second binding site (see chapter 4.2). Since these two compounds differ

from each other by more than one substitution, the effects of the individual substitutions cannot be delineated.

The comparison of **CMS-I-26** and the triazolam analogue **CMS-I-45** demonstrated a comparable potency and efficacy of these compounds at most receptor subtypes (p35). The only difference between these two compounds was that effects at α2 containing receptors were much higher with CMS-I-45 than with CMS-I-26 at concentrations from 100 nM to 10 µM. If the bromo-group is replaced by a much bigger trimethyl-silanyl-ethynyl-group as in compound **CMS-I-59** the potency is reduced. This again seems to be a general feature of this compound series: compounds carrying the big trimethyl-silanyl-ethynyl-group at position 8, such as CMS-I-59 (p35), SH-I-55 (p37), or SH-I-64 (p37) exhibit a reduced potency as compared to compounds substituted with a 8-bromo group (SH-I-85, p38) or 8-ethynyl group (JY-I-59, p39).

4.2 One compound investigated not only interacts with the benzodiazepine binding site but also with an additional binding site present at αβ receptors

To consider the possibility that some of the 12 compounds in addition to the benzodiazepine site also act via another binding site of $GABA_A$ receptors, the effects of these compounds at αβ receptors, which do not contain the benzodiazepine binding site, were investigated too.

All compounds investigated were much more active at αβγ receptors than at αβ receptors and modulation of GABA-induced currents at αβ receptors was weak and only apparent at 10 µM concentrations. At this concentration, only **SH-I-30** showed a stimulation of GABA-induced currents higher than 200% at αβ receptors. Since the other compounds did not stimulate GABA-induced currents at αβ receptors to more than 150% of GABA EC_3 at 10 µM concentrations, it can be assumed that they exhibit most of their action via the benzodiazepine binding site at αβγ receptors.

The 200% stimulation of SH-I-30 at αβ receptors, however, indicates that some of the effects at 10 μM concentrations seen at αβγ receptors do not require a γ2 subunit for receptor stimulation and thus, are not mediated via the classical benzodiazepine binding site, but via a site which is present also at α1β3, α2β3, α3β3, α5β3 receptors.

As the effects of SH-I-30 at a concentration of 1 μM could be inhibited completely by the high affinity benzodiazepine site null modulator Ro 15-1788, we can conclude that 1 μM SH-I-30 mediates all its effects at αxβ3γ2 via the benzodiazepine site.

As it could be shown that on the one hand SH-I-30 at a concentration of 1 μM is exclusively acting via the benzodiazepine site and on the other hand the effects seen at αβ receptors were only present at a concentration of 10 μM, it can be assumed that effects at αβγ receptors up to a concentration of 1 μM result from binding to the benzodiazepine site only. At concentrations higher than 1 μM a second binding site, which is present at αβ receptors, was additionally activated and even increased the GABA-induced currents.

These data supported the assumption that the compound SH-I-30 acts via two different binding sites, on the one hand, via the benzodiazepine site and on the other hand, via another binding site also present at αβ receptors. For further investigations inhibition measurements at 10 μM have to be done.

4.3 Compounds pointing towards α subtype selectivity

Drugs that are affecting preferentially a specific $GABA_A$ receptor subtype are highly interesting because they could elicit some specific behavioral effects in animal experiments and help to unravel the function of the respective receptor subtypes in the brain. To investigate their receptor subtype selectivity, the 12 compounds were investigated at $GABA_A$ receptors containing different α subunits in addition to β3 and γ2 subunits. Many of these compounds were not very strongly stimulating or did not have a preference for one specific receptor subtype, with

some exceptions. Four compounds, however, had the ability to differentially stimulate GABA-induced currents at αβγ receptors containing different α subunits.

SH-I-85 showed already effects in the nM range and selective effects at α2β3γ2, α3β3γ2 and α5β3γ2 receptors at a concentration of 100 nM. The low α1 effect might result in only a small sedative action *in vivo*. Due to its α2/α3/α5 subtype selectivity this benzodiazepine binding site agonist could be important for the treatment of anxiety (Mohler et al., 2001) and schizophrenia (Guidotti et al., 2005; Lewis et al., 2008). This compound is already on the list for behavioral studies in animals.

The compound **JY-I-59** enhanced GABA-induced currents at all receptor subtypes investigated, but showed a strong preference for α3 containing receptors at 10 μM concentration. At lower concentrations, however, this compound could not strongly distinguish between α2/α3/α5 receptors, indicating that it probably would have effects similar to SH-I-85 in *in vivo* experiments.

The already discussed compound **SH-I-30** showed similar enhanced effects at α3/α5 containing receptors at a concentration of 100 nM, whereas stimulation at α1β3γ2 and α2β3γ2 receptors was much lower. This α3/α5 subtype preference could lead to low sedative effects and more anxiolytic activity *in vivo*.

YT-III-19 showed nearly no effects in the nM range and therefore, is not very interesting for further pharmacological investigation. Nevertheless, if the concentration was increased to 10 μM the effects on GABA-induced currents pointed towards an α3/α5 subtype selectivity.

4.3 Conclusion and Outlook

In this study 12 newly synthesized benzodiazepines were investigated for their effects at $GABA_A$ receptors. We found some specific structures, which seemed to be more stimulating and more potent than others, leading to the conclusion that minor changes in their chemical structure could change the efficacy of the compounds. Nevertheless, we cannot propose the ideal structure, because the compounds were too different in total and moreover, the effects have to be

investigated *in vivo*. In addition, the chemical structures of these compounds have to be compared with other existing compounds to broaden the structure-activity relationship. The knowledge of the structure-activity relationship can help to create new ligands for the benzodiazepine binding site.

As a next step, the pharmacological effects of these different benzodiazepines pointing towards subtype selectivity have to be studied *in vivo* and it has to be clarified, whether these different effects at receptor subtypes lead to differences in animal behavior.

If interesting effects are produced by the one or the other compound, the compound might enter experiments leading to a possible clinical application. Such compounds have the potential to become an alternative to conventional BZ site ligands in a clinical setting, because they might exhibit less side effects.

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6 ABBREVIATIONS

BZ	benzodiazepine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC_3	concentration eliciting 3% of maximum effect
EC_{50}	half maximal effective concentration
GABA	γ-aminobutyric acid
$GABA_A$ receptors	GABA type A receptors
GAD	glutamic acid decarboxylase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MB	modified Barth’s medium
mRNA	messenger RNA
RNA	ribonucleic acid
SEM	standard error of the mean
TM	transmembrane
XR	Xenopus Ringer

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