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Positive modulation of synaptic and extrasynaptic $GABA_A$ receptors by an antagonist of the high affinity benzodiazepine binding site

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ABSTRACT

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the brain and are the target for many clinically important drugs such as the benzodiazepines. Benzodiazepines act at the high-affinity binding site at the $\alpha+/\gamma-$ subunit interface. Previously, an additional low affinity binding site for diazepam located in the transmembrane (TM) domain has been described. The compound SJM-3 was recently identified in a prospective screening of ligands for the benzodiazepine binding site and investigated for its site of action. We determined the binding properties of SJM-3 at $GABA_A$ receptors recombinantly expressed in HEK-cells using radioactive ligand binding assays. Impact on function was assessed in *Xenopus laevis* oocytes with electrophysiological experiments using the two-electrode voltage clamp method. SJM-3 was shown to act as an antagonist at the $\alpha+/\gamma-$ site. At the same time it strongly potentiated GABA currents via the binding site for diazepam in the transmembrane domain. Mutation of a residue in M2 of the α subunit strongly reduced receptor modulation by SJM-3 and a homologous mutation in the β subunit abolished potentiation. SJM-3 acts as a more efficient modulator than diazepam at the site in the trans-membrane domain. In contrast to low concentrations of benzodiazepines, SJM-3 modulates both synaptic and extrasynaptic receptors. A detailed exploration of the membrane site may provide the basis for the design and identification of subtype-selective modulatory drugs.

1. Introduction

γ-aminobutyric acid type A ($GABA_A$) receptors are the major inhibitory neurotransmitter receptors in the mammalian central nervous system. These receptors are composed of five homologous subunits that are arranged around a central chloride-selective pore (Macdonald and Olsen, 1994; Barnard et al., 1998; Olsen and Sieghart, 2008; Sigel and Steinmann, 2012). $GABA_A$ receptors belong along with other eukaryotic and procaryotic receptors to the cys-loop pentameric ligand-gated ion channel family (Miller and Smart, 2010). $\alpha_1\beta_2\gamma_2$ is the most prevalent $GABA_A$ receptor isoform in the brain. It consists of $2\alpha_1$, $2\beta_2$, and one γ_2 subunit (Chang et al., 1996; Tretter et al., 1997; Farrar et al., 1999) with a subunit arrangement of $\gamma\beta\alpha\beta\alpha$ anti-clockwise as seen from the synaptic cleft (Tretter et al., 1997; Baumann et al., 2001, 2002; Baur et al., 2006). $GABA_A$ receptors are the target for many clinically important drugs, amongst others the benzodiazepines (Sieghart, 1995). Benzodiazepines act at a high-affinity binding site located at the α_x+/γ_2- ($x = 1, 2, 3, 5$) subunit interface (Sigel and Buhr, 1997; Sigel et al., 1998; Sigel, 2002; Sigel and Lüscher, 2011). Benzodiazepines are positive allosteric modulators that potentiate GABA induced current but do not directly result in appreciable channel opening. They have a large spectrum of clinical actions including sedation, hypnotic effects, anxiolysis, muscle relaxation and anticonvulsant effects.

So far there is evidence for the existence of three different binding sites for benzodiazepines on the $GABA_A$ receptor (Table 1). Apart from the high-affinity benzodiazepine binding site located at the $\alpha+/\gamma-$ subunit interface (called site 1 here), a low-affinity binding site located in a homologous position at the $\alpha+/\beta-$ interface has been postulated (site 2) (Baur et al., 2008). The existence of this site has later been confirmed (Ramerstorfer et al., 2011; Varagic et al., 2013). Earlier in 2000, Walters et al., discovered that in $\alpha_1\beta_2\gamma_2$ receptors potentiation of GABA currents by high concentrations of

Abbreviations: $GABA_A$ receptor, gamma-aminobutyric acid type A receptor; DMSO, dimethylsulfoxide; HEK, human embryonic kidney.

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Table 1
Classification of binding sites.

	Site 1	Site 2[a]	Site 3[b]
Location	α+/γ− subunit interface, classical BZ site	α+/β− subunit interface, homologous to classical BZ site	Intramembrane 2–5 sites at subunit interfaces location see below
Pos. all. mod.	Diazepam (low conc.)	CGS9598	Diazepam (high conc.), Ro15-1788 (high conc.), SJM-3 (high conc.)
Antagonist	Ro15-1788 (low c.), SJM-3	–	–

[a] Site 2 was first described as site for flurazepam and diazepam (intermediate concentration) whose occupation interferes with the action of positive allosteric modulators acting at site 1.
[b] Most probably corresponds to the ivermectin pocket at subunit interfaces seen in GluCl (Hibbs and Gouaux, 2011). It should be noted that ivermectin is a large molecule and the corresponding pocket is even larger.

diazepam results in an additional phase of potentiation. Combined mutation of the homologous residues α_1S269, β_2N265 and γ_2S280 in the second transmembrane domain (M2) each to isoleucine abolished this micromolar component of potentiation while the nanomolar component remained unaffected. Additionally, $\alpha_1\beta_2$ receptors lacking the classical benzodiazepine binding site at the α+/γ− interface were also sensitive to high concentrations of diazepam. The low affinity diazepam site (site 3) was not localized to subunits or subunit interfaces. We tentatively locate it to the huge cavity homologous to the cavity occupied by ivermectin in *Caenorhabditis elegans* GluCl receptors. GluCl S260, homologous to the mutated TM2 positions, forms an H-bond with ivermectin (Hibbs and Gouaux, 2011). The GluCl receptor co-crystallized with ivermectin is homopentameric and thus harbors 5 ivermectin sites, one at each subunit interface. In $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors there are 4 different interfaces each harboring the corresponding cavity.

Recently, we described experiment-guided virtual screening (EGVS), a method that was used to identify new ligands of the classical benzodiazepine binding pocket located at the α+/γ− subunit interface (Middendorp et al., 2014b). It combines selection of homology models in agreement with experimental data on the location of amino acid residues relative to the ligand (Middendorp et al., 2014a) with structure-based virtual screening. Several ligands of the benzodiazepine binding site with a novel scaffold and relatively high affinity have been identified. The compound SJM-3, previously termed compound 3, shown in Fig. 1 rated among the best hits.

Here we show that SJM-3 acts as antagonist at the classical benzodiazepine binding site (site 1) and simultaneously strongly modulates GABA currents via the not precisely located site 3 in the trans-membrane domain previously described by Walters et al. (2000). We also show that many of the novel compounds interacting with site 1 also recognize site 3.

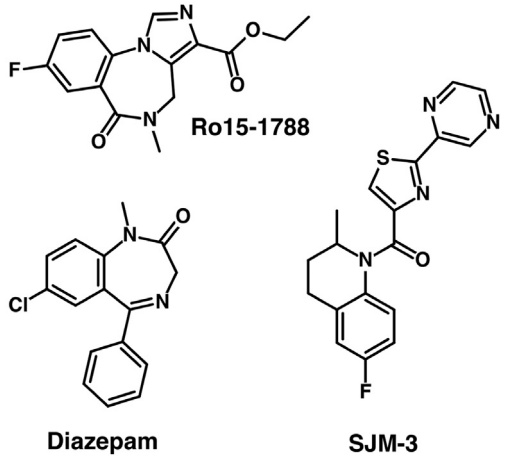

Fig. 1. Chemical structures of diazepam, Ro15-1788 and SJM-3.

2. Methods

2.1. Transfection of $GABA_A$ receptors in HEK293 cells and membrane preparation

The point mutations α_1S269I, β_2N265I and γ_2S280I were prepared using the QuikChange™ mutagenesis kit (Stratagene, Agilent Technologies, Basel, Switzerland). cDNAs coding for wild type or mutated α_1, β_2, and γ_2S subunits of the rat $GABA_A$ receptor were transfected in human embryonic kidney (HEK) 293 cells (American Type of Culture Collection, MD, USA, CRL 1573) at a ratio of 1:1:1 with 20 μg DNA per 90 mm dish. Culturing of cells and membrane preparation was carried out as before (Tan et al., 2007).

2.2. Radioactive ligand binding assay

Ligand binding assays were performed as described before (Baur et al., 2010). Briefly, resuspended cell membranes (360 μL) were incubated for 60 min on ice in the presence of [^{3}H]-Ro15-1788 (85.4 Ci mmol^{-1}, PerkinElmer) or [^{3}H]-flunitrazepam (82.5 Ci mmol^{-1}, PerkinElmer). Membranes were collected by rapid filtration on GF/C filters (Macherey-Nagel, Germany). After three washing steps with 5 mL of buffer, the filter-retained radioactivity was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 20 μM Ro15-1788 or flunitrazepam, respectively. Displacement assays were carried out with [^{3}H]-Ro15-1788 or [^{3}H]-flunitrazepam as radioactive ligand and increasing concentrations of SJM-3. K_d values for [^{3}H]-Ro15-1788 and [^{3}H]-flunitrazepam have previously been reported to be 0.7 and 2.0 nM, respectively (Baur et al., 2010). The concentration of [^{3}H]-Ro15-1788 and [^{3}H]-flunitrazepam used in the displacement assays was about 2 times the K_d value. On the basis of IC_{50} determinations, the K_i value was determined according to the Cheng–Prusoff equation (Cheng and Prusoff, 1973). Data were fitted by using a nonlinear least-squares method to the equation $B(c) = B_{max}/(1 + K_d/c)$ for binding curves and $B(c) = B_{max}/(1 + (c/IC_{50}))$ for displacement curves, where c is the concentration of ligand, B, binding, B_{max}, maximal binding, K_d, dissociation constant, and IC_{50}, concentration of half-maximal inhibition. In case no complete inhibition was reached in the displacement experiments the data were fitted to the equation $(B_{max} - \text{Bottom})/(1 + (c/IC_{50})) + \text{Bottom}$. All fitting procedures were performed with the Kaleidagraph software. In all experiments each point was determined in triplicate.

2.3. Expression and functional characterization in Xenopus oocytes

Capped cRNAs were synthesized (Ambion, Austin, TX, USA) from the linearized plasmids with a cytomegalovirus promotor (pCMV vectors) containing the different subunits, respectively. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase (United States Biologicals, Cleveland, OH, USA). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad) for visualization of the RNA. Known concentrations of RNA ladder (Invitrogen) were loaded as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19:1, the dried pellet dissolved in water and stored at −80 °C. cRNA mixtures were prepared from these stock solutions and stored at −80 °C.

Xenopus laevis oocytes were prepared, injected and defolliculated as described previously (Sigel, 1987; Sigel and Minier, 2005; Animal research permit by the Kantonstierarzt, Kantonaler Veterinärdienst Bern (BE98/12)). They were injected with 50 nL of the cRNA solution containing wild type or mutated rat α_1, β_2 and γ_2S subunits of the $GABA_A$ receptors at a concentration of 10 nM:10 nM:50 nM (Boileau et al., 2002) and then incubated in modified Barth's solution at 18 °C for at least 24 h before the measurements.

Currents were measured using a modified two-electrode voltage clamp amplifier Oocyte clamp OC-725 (Warner Instruments) in combination with a XY-recorder (90% response time 0.1 s) or digitized at 100 Hz using a PowerLab 2/20 (AD