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Actions of two $GABA_A$ receptor benzodiazepine-site ligands that are mediated via non-γ2-dependent modulation

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ABSTRACT

The potent sedative-hypnotic zolpidem and the convulsant methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) act primarily by binding to the benzodiazepine site of the main inhibitory neurotransmitter receptor, the pentameric γ-aminobutyric acid type A receptor ($GABA_A$). This binding depends critically on the wild-type F77 residue of the $GABA_A$ receptor γ2 subunit. Mice with γ2 subunit F77I point mutation (γ2I77 mouse line) lose the high-affinity nanomolar binding of these ligands as well as their most robust behavioral actions at low doses. Interestingly, the γ2I77 mice offer a tool to study the actions of these substances mediated via other possible binding sites of the $GABA_A$ receptor. In ligand autoradiographic experiments, we discovered in γ2I77 mouse brain sections a significant amount of residual non-γ2 subunit-dependent benzodiazepine site binding enriched to the striatum and septum. Zolpidem only weakly affected this residual binding at micromolar concentrations, and only a high zolpidem dose (≥40 mg/kg) caused sedation and deficits in motor coordination in γ2I77 mice. DMCM had an agonistic action through a secondary, low-affinity non-benzodiazepine binding site of the $GABA_A$ receptor in the forebrain of γ2I77 mice, and this drug also fully displaced the residual benzodiazepine-site labeling. In behavioral tests, a high dose (20 mg/kg) of DMCM was sedative and modulated fear learning. DMCM, but not zolpidem, acted as an agonist in recombinant $GABA_A$ α1/6β3 receptors studied using ligand binding and electrophysiological assays. Our results highlight the less well-known actions of high doses of DMCM and zolpidem that are not mediated via the γ2 subunit-containing benzodiazepine site of the $GABA_A$ receptor.

1. Introduction

Classical benzodiazepines, rather non-selective for the subtype of the pentameric $GABA_A$ receptor they activate, produce a variety of clinical effects, including hypnosis, anxiolysis, myorelaxation, sedation, amnesia, tolerance and dependence. On the other hand, based on genetically modified mouse models, many of the actions of benzodiazepines are mediated by specific $GABA_A$ receptor subtypes, e.g. sedation and amnesia by α1 subunit-containing receptors (Rudolph et al., 1999), anxiolysis and myorelaxation by α2 and α3 subunit-containing receptors (Crestani et al., 2001; Low et al., 2000), and learning impairment and tolerance by α5 subunit-containing receptors (Charlton et al., 1997; Collinson et al., 2002). Receptors containing α4 and α6 subunits seem to partly mediate the actions of non-benzodiazepine compounds like gaboxadol and neurosteroids (for review, see Lambert et al., (1996)). By characterizing various $GABA_A$ receptor binding sites, new treatment modalities may be discovered and fine-tuned without the adverse effects of present drugs.

The benzodiazepine binging pocket is situated on the interface of the α and γ subunits of the $GABA_A$ receptor (Buhr and Sigel, 1997), and point mutations at or near this site affect the ability of the pocket to bind benzodiazepines and benzodiazepine-like substances (Buhr and Sigel, 1997; Teissere and Czajkowski, 2001; Whiting et al., 1999; Wieland et al., 1992). We have engineered a knockin mouse line with an F77I point mutation in the γ2 subunit gene (γ2I77 mouse line), rendering these mice behaviorally insensitive to low doses of zolpidem and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) that induce in wild-type mice strong sedative-hypnotic effects (Cope et al., 2004) and convulsions (Leppa et al., 2005), respectively. γ2I77 mice retain normal sensitivity to classic benzodiazepines, such as diazepam. The affinities of zolpidem and DMCM to the benzodiazepine-site of $GABA_A$ receptors in forebrain membranes

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of γ2I77 mice are reduced by 300 and 1700 fold, respectively (Ogris et al., 2004), as compared to wild-type receptors, with the K_i values for inhibition of the binding of [^{3}H]flunitrazepam by zolpidem and DMCM being as high as 30 and 9 μM, respectively, in the γ2I77 receptors. Several GABAergic drugs have another, low-affinity binding/effector site in the $GABA_A$ receptor (Walters et al., 2000), which, interestingly, is common to DMCM (Thomet et al., 1999) and also to anesthetic/anticonvulsive substances like etomidate and loreclezole (Stevenson et al., 1995; Wafford et al., 1994). DMCM has a positively modulating effect at this binding site (Stevenson et al., 1995), but its significance has not been clarified.

We now studied a possible affinity of zolpidem to the $GABA_A$ receptor by quantitative ligand autoradiography of γ2I77 mouse brains and by administering high doses of the drug to γ2I77 mice in behavioral tests. We also have characterized the positive modulation of the $GABA_A$ receptor by DMCM in the γ2I77 mouse line, using behavioral sensitivity tests, and mapped the apparent residual drug effects using ligand autoradiography. In addition, we have tested recombinant αβ $GABA_A$ receptors for the effects of zolpidem and DMCM in order to simulate their efficacy at receptors without the γ2 subunit.

2. Materials and methods

2.1. Experimental animals

Generation of the γ2I77 mouse line is described elsewhere (Cope et al., 2004). Male and female γ2I77 mice (bred in-house, n = 79) and C57BL/6J wild-type (WT) mice (bred in-house, n = 25), aged 4–12 months (20–45 g), were used. The animals were housed (1–5 per cage) in transparent polypropylene Makrolon cages (37 × 21 × 15 cm; Tecniplast, Buguggiate, Italy) with standard rodent pellets (Harlan Teklad Global Diet, Bicester, UK) and tap water ad libitum. Temperature was controlled at 21 °C. Lights were on from 6 a.m. to 6 p.m. All animal experiments were approved by the Southern Finland Provincial Government and the Institutional Animal Use and Care committee of the University of Helsinki. All efforts were made to minimize the number and suffering of animals used.

The γ2I77 mouse line is phenotypically similar to wild-type littermate and C57BL/6J control mice (Cope et al., 2004). DMCM doses below 20 mg/kg (Leppä et al., unpublished results) and zolpidem doses below 30 mg/kg (Cope et al., 2004) have no discernible behavioral effect on γ2I77 mice. Zolpidem was not administered to C57BL/6J control mice in our experiments (except for electroshock protection experiments), because already the dose of 6 mg/kg induces heavy sedation, sleep and an inability to stay even on an unmoving rotarod (Cope et al., 2004). Neither was DMCM administered to C57BL/6J mice in this study because even low 1–3 mg/kg doses of this benzodiazepine-site inverse agonist may cause convulsions in wild-type animals (Braestrup et al., 1982; Tsuda et al., 1997).

2.2. Determination of benzodiazepine-site binding by [^{3}H]Ro 15-4513 autoradiography in brain sections

Autoradiography of male and female γ2I77 (n = 5) and C57BL/6 (n = 5) brain sections was performed as described earlier (Mäkelä et al., 1997). Mice were decapitated and whole brains were dissected and frozen on dry ice. Coronal 14-μm thick serial sections were cut with a cryostat (Leica CM3050 S, Leica Microsystems, Nussloch, Germany) and mounted on gelatin-coated object glasses. The sections were frozen and stored (−80 °C) with a desiccant (silica gel).

Cryostat sections were preincubated for 15 min in an ice-water bath in incubation buffer [50 mM Tris–HCl, 120 mM NaCl (pH 7.4)]. The final incubation took place with 15 nM [^{3}H]Ro 15-4513 (Perkin-Elmer Life Sciences Inc., Waltham, MA, USA) in the incubation buffer in the dark at 4 °C for 60 min in plastic slide mailers. Effects of 0.01–10 μM zolpidem and 0.01–10 μM DMCM on radioligand binding were determined. Nonspecific binding was determined with 10 μM Ro 15-1788 (flumazenil). After incubation, the sections were washed with ice-cold incubation buffer for 2 × 60 s, dipped in distilled water and dried in air flow at room temperature. The sections were then exposed with [^{3}H]-plastic standards (GE Healthcare, Little Chalfont, Buckinghamshire, UK) to BAS-TR 2040 imaging plate (Fujifilm Corporation, Tokyo, Japan) for 14 days or to Kodak Biomax MR films (Eastman Kodak, Rochester, NY) for 6 months. Representative images from autoradiography films were scanned using an EPSON Expression 1680 Pro scanner and EPSON Scan v. 1.11e program (Epson corporation, Long Beach, CA, USA) and finalized in CorelDraw X3 (Corel Corporation, Ottawa, Canada). For quantification of binding densities, the imaging plates were first scanned by the FLA-9000 Starion image scanner (Fujifilm) and then analyzed with Scion Image analysis program (Scion Corporation, Frederick, Maryland) or ImageJ analysis program (ImageJ 1.43U, NIH, Maryland, US). Binding densities for each brain area were referenced to the [^{3}H]-standards, converted to radioactivity levels estimated for gray matter areas (nCi/mg), and given as means ± S.E.M.

2.3. Determination of allosteric effects on GABA modulation by [^{35}S]TBPS autoradiography of brain sections

[^{35}S]*t*-Butylbicyclophosphorothionate ([^{35}S]TBPS) is a widely used $GABA_A$ receptor radioligand, the binding of which can be modulated by various substances that either increase or decrease its affinity to the ion channel pore of the receptor (Squires and Saederup, 1993). GABA, and substances that enhance the actions of GABA, decrease [^{35}S]TBPS binding, whereas substances that inhibit or decrease the actions of GABA produce the reverse effect. Autoradiography of male and female γ2I77 (n = 5) and C57BL/6J (n = 5) brain sections was performed as described earlier (Mäkelä et al., 1997).

Cryostat sections were preincubated for 3 × 10 min in an ice-water bath in 50 mM Tris–HCl (pH 7.4). One mM EDTA (Sigma Aldrich Chemical Company, St. Louis, MO, USA) was added to the preincubation buffer to remove endogenous GABA (Mäkelä et al., 1997). The sections were dipped in the incubation buffer to wash out EDTA before the final incubation. The final incubation took place with 6 nM [^{35}S]TBPS (about 200 cpm/μl) (Perkin-Elmer) in the incubation buffer (50 mM Tris–HCl, 120 mM NaCl, pH 7.4) at room temperature for 90 min. The effects of 1 and 10 μM zolpidem and 1 and 30 μM DMCM were investigated in the presence of 2 μM GABA. Radioactivity of the incubation solution was ascertained with a liquid scintillation counter (1217 RackBeta, LKB Wallac, Perkin-Elmer). Nonspecific binding was determined with 100 μM picrotoxinin (Sigma). After the incubation, the sections were washed with buffer (10 mM Tris–HCl, 120 mM NaCl, pH 7.4) for 3 × 2 min, dipped in distilled water and dried in air flow at room temperature. The sections were then exposed with [^{14}C]-plastic standards (GE Healthcare, Little Chalfont, Buckinghamshire, UK) to BAS-MS 2040 imaging plate (Fujifilm Corporation, Tokyo, Japan) for 6 h or to Kodak Biomax MR films (Eastman Kodak, Rochester, NY) for 2 weeks. Representative images from autoradiography films and quantification of binding densities were carried out as described above. Binding densities for each brain area were referenced to the standards, converted to radioactivity levels estimated for gray matter areas (nCi/g), and given as means ± S.E.M.

2.4. Simulation using recombinant receptors

2.4.1. Culturing and transfection of HEK 293 cells for electrophysiology and binding assays

For electrophysiological recording HEK 293 cells were passaged and replated on 12-mm glass coverslips located in 9.6-cm plastic dishes. For binding assays 15-cm plates were employed (digits given