Enhancing Neurosteroid Synthesis – Relationship to the Pharmacology of Translocator Protein (18 kDa) (TSPO) Ligands and Benzodiazepines

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Key words
- TSPO ligands
- benzodiazepines
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Abstract

Introduction: The treatment of anxiety disorders is still a challenge; novel pharmacological approaches that combine rapid anxiolytic efficacy with fewer side effects are needed. A promising target for such compounds is the mitochondrial translocator protein (18 kDa) (TSPO). TSPO plays an important role for the synthesis of neurosteroids, known to modulate $GABA_A$ receptors, thereby exerting anxiolytic effects.

Methods: We investigated the pharmacological profile of 2 well established TSPO ligands (XBD173 and etifoxine) compared to the benzodiazepine diazepam with regard to TSPO binding affinity, TSPO expression and neurosteroidogenesis.

Results: In BV-2 microglia and C6 glioma cells all compounds significantly enhanced TSPO protein expression. Radioligand binding assays revealed the highest binding affinity to TSPO for XBD173, followed by diazepam and etifoxine. Pregnenolone synthesis was most potently enhanced by etifoxine.

Discussion: Etifoxine turned out to be the most potent enhancer of neurosteroidogenesis, although its binding affinity to TSPO was lowest. These results indicate that the efficacy of TSPO ligands to stimulate neurosteroid synthesis, thereby leading to anxiolytic effects cannot be concluded from their binding affinity to TSPO.

Introduction

Mental disorders are widespread and highly disabling with a considerable socioeconomic impact. Among these, anxiety disorders belong to the most frequent. A 2004 WHO commissioned study of EU citizens found that 13.6% of the population suffer from these disorders [1]. However, the pharmacological treatment of anxiety disorders is still a challenge [2]. First line treatment options in acute states are benzodiazepines (e.g., diazepam). These drugs have a rapid onset of action, but they are sedative and quickly induce tolerance and abuse liability. For long-term treatment, certain antidepressants, such as selective-serotonin-reuptake-inhibitors (SSRIs) are preferred because they are not sedative and do not cause tolerance. However, these drugs have a delayed onset of action of several weeks. It is obvious that novel compounds for the treatment of anxiety disorders are needed that combine rapid anxiolytic efficacy with a preferable side effect profile.

Currently, one of the most promising novel drug targets for the treatment of anxiety disorders is the translocator protein 18 kDa (TSPO) [3]. TSPO is a 5 helical transmembrane protein located in the outer mitochondrial membrane. It promotes the transport of cholesterol into the mitochondrial matrix, which is the rate-limiting step in neurosteroid synthesis. Cholesterol is then metabolized to pregnenolone by CYP 11A1 (cholesterol side-chain cleavage enzyme, P450scc). Pregnenolone is then further metabolized to other neurosteroids, such as allopregnanolone and 3α,5α-THDOC (3alpha,5alpha-tetrahydrodeoxycorticosterone) which are potent modulators of $GABA_A$ receptor function, thereby exerting anxiolytic effects [3]. TSPO is expressed in many organs, the highest expression levels are found in steroid-synthesizing cells, e.g., adrenal, gonad and brain cells [4]. In the central nervous system (CNS), TSPO is mainly expressed in microglia [5] and in reactive astrocytes [6].

Most TSPO ligands are primarily used in neuroimaging as diagnostic tools for brain inflammation [7]. However, some TSPO ligands already

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Bibliography
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play a role in the pharmacological treatment of anxiety disorders. The benzoxazine etifoxine was the first TSPO ligand that showed clinical anxiolytic effects comparable to those of the benzodiazepine lorazepam in patients suffering from adjustment disorders with anxiety [8]. Etifoxine (trade name Stresam) is used as an anxiolytic and anticonvulsant. However, this drug is also a weak direct $GABA_A$ receptor modulator [9]. The later developed compound XBD173 (AC-5 216/emapunil) is a phenylpurine that is rather selective for TSPO; it has also been shown to exert potent anxiolytic efficacy in animals as well as in humans without causing sedation [10]. Because the proof of concept study with XBD173 using the cholecystokinin tetrapeptide (CCK-4) challenge paradigm in healthy volunteers was positive, a phase II trial in patients with generalized anxiety disorder was conducted which, however, did not show superiority over placebo (unpublished data). Diazepam is a frequently prescribed anxiolytic belonging to the group of benzodiazepines, which are direct $GABA_A$ receptor modulators. They bind to the benzodiazepine binding site (located at the interface of α and γ subunits) thereby increasing the frequency of the chloride ion channel opening in the presence of GABA [11]. However, some benzodiazepines have also been reported to bind to TSPO [3].

In summary, there is a considerable overlap between different classes of anxiolytic compounds with regard to their pharmacological mechanisms of action. However, their clinical effects and also side effects differ notably. To get a better insight into the pharmacology of these drugs we characterized the mechanism of action of the TSPO ligands XBD173 and etifoxine in comparison to the benzodiazepine diazepam with regard to TSPO binding affinity, TSPO expression and neurosteroid production.

Materials and Methods

Chemicals

Etifoxine and diazepam were purchased from Sigma-Aldrich (Seelze, Germany). XBD173 was obtained by custom synthesis (APAC Pharmaceuticals, Columbia, USA). All compounds were dissolved in ethanol as stock solutions of 10 mM.

Cell culture

BV-2 mouse microglia cells were a generous gift from Dr. Marcus Karlstetter (Center of Ophtalmology, Experimental Immunology of the Eye, University of Cologne, Germany). C6 rat glioma cells were a generous gift from Dr. Barbara Di Benedetto (Max-Planck-Institute of Psychiatry, Munich). BV-2 cells were cultured in Roswell Park Memorial Institute Medium (RPMI 1640 Medium, PAA Laboratories, Cölbe, Germany) supplemented with 5% fetal calf serum (FCS), 1% L-glutamine and 195 nM mercaptoethanol. C6 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% FCS and 1% sodium pyruvate. All media were supplemented with 1% (v/v) penicillin-streptomycin. Cells were cultured at 37 °C, 95% humidity and 5% CO_2.

Protein lysates and drug treatment

BV-2 and C6 cells were seeded onto 6-well culture plates until 80% confluence. Cells were incubated for 24 h with XBD173, etifoxine or diazepam at concentrations of 0.1 µM, 1 µM, 3 µM and 10 µM, respectively. These concentrations were chosen according to concentrations reached in the cerebrospinal fluid of humans treated with diazepam [12]. The compounds were added directly to the culture medium. Solvent (ethanol) concentration was 0.5% in each well. After 24 h cells were harvested by scrapping cells in 1 mL HEPES buffer [20 mM HEPES, 5 mM EDTA, 1 M NaCl supplemented with a protease inhibitor cocktail (Sigma-Aldrich)] and subsequent sonication. Lysates were stored at −20 °C until further use.

Western blotting

Protein concentrations of cell lysates were quantified by means of the Bradford method [13] with the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Munich, Germany). 10 µg of protein lysates were separated by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) with 15% polyacrylamide gels and were then transferred onto a nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in TBST (10 mM TRIS, 150 mM NaCl, 0.1% Tween 20; pH 7.4). TSPO was detected with a goat-anti-TSPO antibody (My Biosource, San Diego, CA, USA) and ß-actin with a rabbit-anti-ß-actin (Sigma-Aldrich) overnight at 4 °C. Blots were washed with TBST and incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies. Bands were detected with the Chemiluminescence Substrate Super Signal West Pico (Fisher Scientific, Schwerte, Germany) and visualized with a digital imaging system (Image Quant LAS 4000, GE Healthcare Europe, Freiburg, Germany). Densitometry analysis was performed with ImageJ Software (Wayne Rasband, National Institute of Health, USA). TSPO values were normalized to ß-actin values of the same sample and to control (cells treated with solvent).

Pregnenolone ELISA

BV-2 and C6 cells were seeded onto 24-well plates. 80% confluent cells were treated for 21 h with etifoxine, XBD173 or diazepam directly added to the medium at concentrations of 0.1 µM, 1 µM, 3 µM and 10 µM, respectively. These concentrations were chosen according to concentrations reached in the cerebrospinal fluid of humans treated with diazepam [12]. After this first period of incubation plates were washed once with phosphate buffered saline (PBS, Sigma-Aldrich) and then filled with 1 mL pregnenolone assay buffer (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose and 10 mM HEPES) [14] supplemented with 0.1% BSA (bovine serum albumin, Sigma-Aldrich) and 25 µM trilostane (Sigma-Aldrich). Cells were treated again with the above mentioned compounds for further 3 h. Afterwards, supernatants were used in an enzyme-linked immunosorbent assay for pregnenolone quantification, according to the manufacturer's recommendations (Pregnenolone ELISA, IBL International, Hamburg, Germany). In brief, 2 × 50 µL of each sample were pipetted on a rabbit anti-pregnenolone antibody coated 96-microwell plate. 100 µL of pregnenolone-HRP conjugate was then added. Ready-to-use-calibrators were provided by IBL International. Afterwards 150 µL of tetramethylbenzidine/hydrogen peroxide (TMB) substrate were added. After 15 min, 50 µL of stopping solution were pipetted into each well. Assays were read with a Tecan Spectra photometer (Crailsheim, Germany) at 450 nm. Data were analyzed by Magellan Data Analysis Software (Tecan, Version 2.0). Pregnenolone concentrations were normalized to control (cells treated with solvent).

[^{3}H]PK11195 radioligand binding assay

[^{3}H]PK11195 saturation binding assays were performed using cell lysates of BV-2 and C6 cells, prepared as previously described.

Lysates were rehomogenized with assay buffer (50 mmol/L TRIS; pH = 7.4). Lysates were pipetted into a 96-well plate at a final volume of 100 µL containing 35 µg total protein per well. Saturation binding assays included the following concentrations: 0.1 nM, 0.5 nM, 1 nM, 2 nM, 3 nM, 4 nM, 5 nM and 6 nM of [^{3}H]PK11195 (Perkin Elmer, Downers Grove, IL, USA) with a specific activity of 82–83–83 Ci/mmol. Samples were incubated with 10 µmol/L unlabelled PK11195 (Sigma-Aldrich). Competitions were performed with [^{3}H]PK11195 at a concentration of 1 nM for each sample. The competitors etifoxine, XBD173 or diazepam were added at concentrations of 0.1 nM, 0.5 nM, 1 nM, 5 nM, 10 nM, 15 nM and 20 nM, respectively. The concentrations of competing compounds were chosen according to the K_d value of [^{3}H]PK11195, which had a high binding affinity already at nanomolar concentrations (data not shown). Assays were incubated at 25 °C for 60 min. Afterwards assays were terminated by vacuum filtration through a UniFilter-96 GF/C with 1.2 µm pore size (PerkinElmer) with ice-cold assay buffer. The amount of bound [^{3}H]PK11195 was determined using a Mikroszint 20 (Perkin Elmer) and the Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer). Data (disintegration per minute) were transformed to fmol [^{3}H]PK11195 bound per mg protein (fmol/mg). Specific binding values were calculated as the difference between total and non-specific binding measured in triplicate for each condition. Saturation binding data were analysed by non-linear regression with GraphPad Prism 5.0 (San Diego, CA, USA). B_{max} and K_D values, respective measures of [^{3}H]PK11195 binding capacity (indicating PBR/TSPO density), were also measured with Graph Prism 5.0.

WST-1 test

The cell proliferation reagent WST-1 test (Roche Diagnostics, Mannheim, Germany) was used for spectrophotometric quantification of cell proliferation and viability to quantify cytotoxicity of drugs. BV-2 and C6 cells were treated for 24 h with XBD173, etifoxine or diazepam at concentrations of 0.1 µM, 1 µM, 3 µM and 10 µM respectively. These concentrations were chosen according to concentrations reached in the cerebrospinal fluid of humans treated with diazepam [12]. The compounds were added directly to the culture medium. Solvent (ethanol) concentration was 0.5% in each well. After 24 h, cell medium was changed in color free Opti-MEM (Life Technologies GmbH, Darmstadt, Germany) and samples were incubated with WST-1 reagent for 3.5 h at 37 °C. Assays were read with a Tecan Spectra Reader at 450 nm. Values were normalized to control (solvent).

Statistical analysis

Data are presented as mean ± SEM of at least 6 independent experiments. For the comparison of mean differences in TSPO expression and pregnenolone synthesis between a single treatment compared to control Student's t-tests was used. One-way analysis of variance (ANOVA) was performed to detect whether there are significant differences in TSPO expression and pregnenolone synthesis between the different compounds and dosages. In case of multiple testing Bonferroni correction was used. As a nominal level of significance, $\alpha = 0.05$ was accepted. Statistical analyses were performed using SPSS for Windows (Release 20, SPSS Inc., Chicago, IL 60606, USA), version 18.0.3 and IBM SPSS Statistics (SPSS Inc.), version 19.0.1.

Results

TSPO binding affinity

To determine the binding affinity of XBD173, etifoxine and diazepam, we performed competitive [^{3}H]PK11195 radioligand binding assays and measured concentration-dependent displacement curves. The binding affinity of each ligand is stated as IC_{50}, which shows the concentration of a drug at 50% of [^{3}H]PK11195 displacement. IC_{50} values appeared sufficient to compare binding affinities of these TSPO ligands, because experimental conditions were identical for all 3 compounds under investigation. As shown in **Fig. 1a** for BV-2 mouse microglia cells, XBD173 was the TSPO ligand with the highest binding

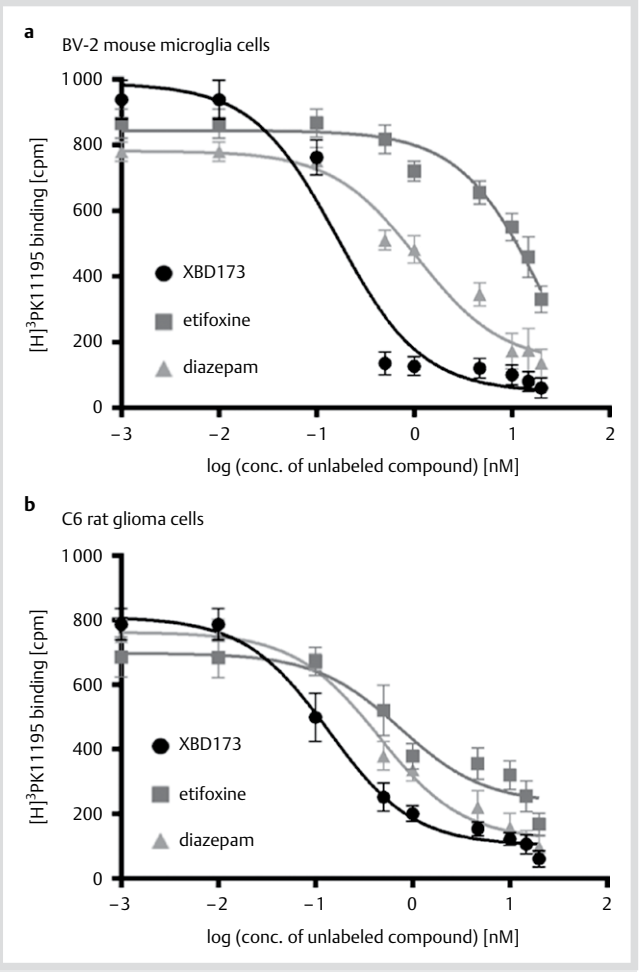

Fig. 1 [^{3}H]PK11195 radioligand binding assay. Displacement of [^{3}H]PK11195 binding to TSPO by XBD173, etifoxine, or diazepam. Binding assays were conducted with 1 nM [^{3}H]PK11195. The competitors XBD173, etifoxine, or diazepam were added at concentrations of 0.1 nM, 0.5 nM, 1 nM, 5 nM, 10 nM, 15 nM and 20 nM, respectively. The amounts of bound [^{3}H]PK11195 were determined using a Mikroszint20 and the Tri-Carb Scintillation Analyzer. Data were analysed with GraphPad Prism 6.0 and represent the mean values of 3 independent experiments (each measured in triplicate). The competition of [^{3}H]PK11195 binding by increasing concentrations of incubated compounds in logarithmic representation is shown. **a** BV-2 mouse microglia cells: IC50 XBD173: 0.16 nM (95% confidence interval ~ 0.09 nM to 0.30 nM), IC50 etifoxine: 22.78 nM (95% confidence interval ~ 4.68 nM to 111.00 nM), IC50 diazepam: 1.07 nM (95% confidence interval ~ 0.58 nM to 1.98 nM). **b** C6 rat glioma cells: IC50 XBD173: 0.14 nM (95% confidence interval ~ 0.09 nM to 0.22 nM), IC50 etifoxine: 0.75 nM (95% confidence interval ~ 0.29 nM to 1.92 nM), IC50 diazepam: 0.45 nM (95% confidence interval ~ 0.27 nM to 0.73 nM).

affinity (IC_{50} of 0.16 nM). Diazepam showed an intermediate IC_{50} of 1.07 nM and etifoxine had the lowest binding affinity reflected by an IC_{50} of 22.78 nM. Also in C6 rat glioma cells (**Fig. 1b**) XBD173 showed the highest binding affinity with an IC_{50} of 0.14 nM, diazepam was intermediate with an IC_{50} of 0.45 nM and etifoxine with the highest IC_{50} of 0.75 nM had the lowest binding affinity to TSPO. However, the range of differences between the IC_{50} values of the TSPO ligands was higher in microglial cells (BV-2) than in astrocytes (C6).

TSPO protein expression

To investigate changes of TSPO protein expression by TSPO ligands C6 and BV-2 cells were treated with XBD173, etifoxine or diazepam as described previously. The effect of 24 h drug incubation on TSPO protein expression in BV-2 cells is presented in **Fig. 2a, b**. All compounds significantly increased TSPO protein expression already at the lowest concentration applied (Student's t-test for all compounds and all concentrations $p < 0.05$ compared to untreated cells, respectively). However, there were no significant concentration-related effects on TSPO expression by XBD173, etifoxine or diazepam treatment (ANOVA: $F = 1.452$, $df = 3, 230$, $p = 0.228$). Moreover, there was no significant difference between the different drugs on TSPO protein expression (ANOVA: $F = 1.284$, $df = 2, 230$, $p = 0.279$) and no significant interaction effect between the different compounds and concentrations used (ANOVA: $F = 0.728$, $df = 6, 230$, $p = 0.627$).

The effects of TSPO ligands on TSPO protein expression in C6 were comparable to those in BV-2 cells as shown in **Fig. 3a, b**. Also in C6 cells, all compounds under investigation significantly increased TSPO protein expression already at the lowest concentration applied (Student's t-test). Similarly, there were no statistically significant differences in TSPO protein upregulation with regard to the different concentrations of drugs (ANOVA: $F = 2224$, $df = 3, 246$, $p = 0.086$) or between the different compounds (ANOVA: $F = 1350$, $df = 2, 246$, $p = 0.261$). There was also no interaction effect between compounds and concentrations applied (ANOVA: $F = 0.664$, $df = 6, 246$, $p = 0.679$).

Within the concentration ranges used in this study (0.1 µM to 10 µM), all drugs under investigation did not show antiproliferative or toxic effects (data not shown).

Neurosteroid synthesis

Because the potential anxiolytic efficacy of a TSPO ligand is crucially determined by its potency to enhance neurosteroid synthesis, pregnenolone levels after treatment with XBD173, etifoxine or diazepam were measured in cell culture supernatants by means of ELISA. Effects of drug incubation on pregnenolone synthesis in BV-2 cells are presented in **Fig. 4a**. Etifoxine turned out to be the most potent compound, which significantly upregulated pregnenolone levels in a dose-dependent fashion (ANOVA: $F = 9471$, $df = 3, 95$, $p < 0.001$). However, upon treatment with the highest concentration (10 µM), there was a marked drop of pregnenolone synthesis. Also XBD173 showed a significant enhancement of pregnenolone synthesis, which was less pronounced and showed a weaker, nonetheless significant dose-response relationship (ANOVA: $F = 3247$, $df = 3, 95$, $p = 0.025$) with a peak at 3 µM. Diazepam did not show a significant enhancement of pregnenolone synthesis at all concentrations under investigation (Student's t-test for all concentrations $p > 0.05$ compared to untreated cells, respectively).

The effects of TSPO ligands on pregnenolone synthesis in C6 cells are shown in **Fig. 4b**. Similar to BV-2 cells, etifoxine promoted pregnenolone synthesis most potently and in a dose-dependent manner (ANOVA: $F = 12999$, $df = 3, 58$, $p < 0.001$). The effect of XBD173 was even less pronounced in C6 cells, but with a considerable concentration effect (ANOVA: $F = 82179$, $df = 3, 58$, $p < 0.001$). However, only the highest concentration applied significantly upregulated pregnenolone synthesis compared to control (Student's t-test: $p = 0.005$). Diazepam showed a marked upregulation of pregnenolone synthesis at a concentration of 1 µM (Student's t-test: $p < 0.001$) and 10 µM (Student's t-test: $p < 0.001$). Summing up, etifoxine was significantly more potent in enhancing pregnenolone synthesis in C6 cells compared to XBD173 and diazepam ($p < 0.001$), whereas there was no marked difference between XBD173 and diazepam ($p = 0.344$) as shown by Bonferroni-corrected post-hoc tests.

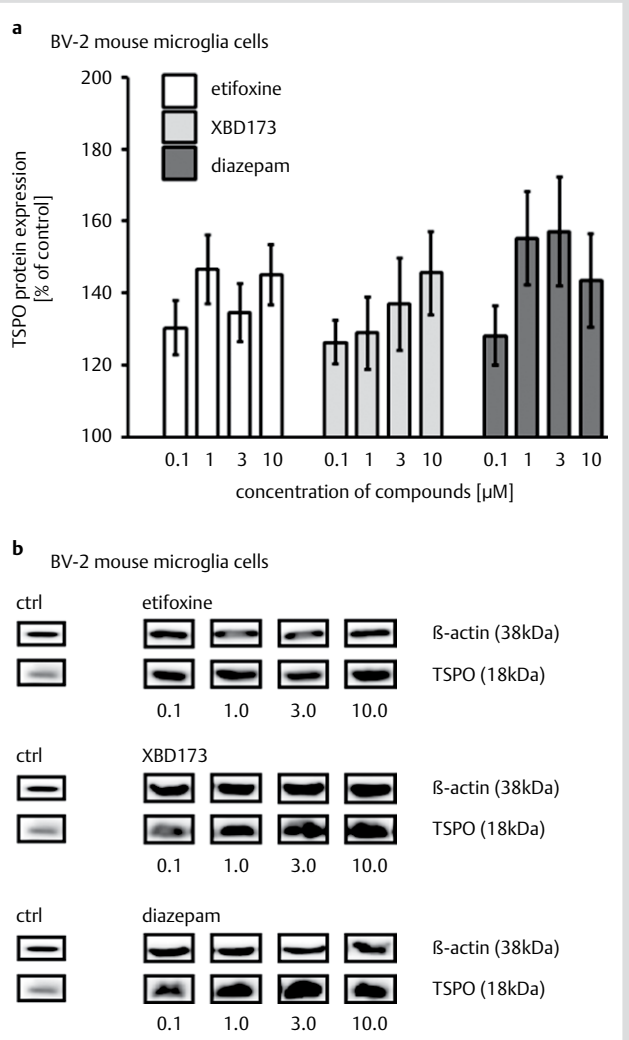

Fig. 2 TSPO protein expression in BV-2 mouse microglia cells. Increase of TSPO protein expression in BV-2 cells after incubation with XBD173, etifoxine, or diazepam. Cells were treated with the respective compounds at different concentrations: 0.1 µM, 1 µM, 3 µM and 10 µM. After 24 h, cells were harvested and protein levels of cell lysates were determined by means of Bradford assay. Equal amounts of protein were separated by SDS-PAGE. Specific antibody binding against TSPO and ß-actin was detected by chemiluminescence. **a** Densitometrical analysis of TSPO expression of at least 6 independent experiments. Data are presented as mean ± SEM % of control (cells treated with solvent). **b** One representative experiment is shown. Ctrl: control.

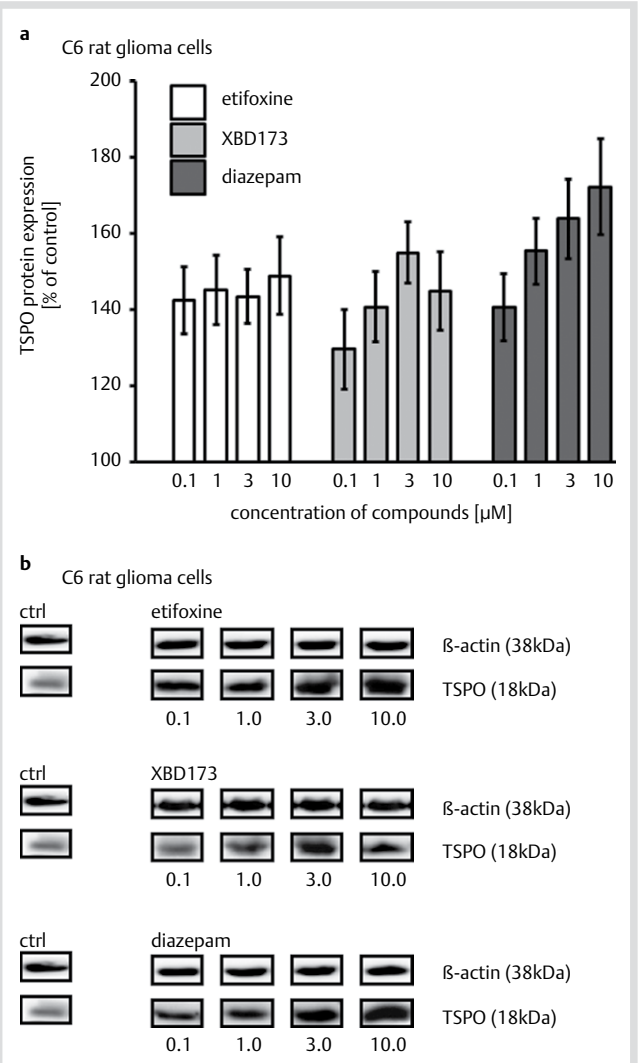

Fig. 3 TSPO protein expression in C6 rat glioma cells. Increase of TSPO protein expression in C6 cells after incubation with XBD173, etifoxine, or diazepam. Cells were treated with the respective compounds at different concentrations: 0.1 µM, 1 µM, 3 µM and 10 µM. After 24 h, cells were harvested and protein levels of cell lysates were determined by means of Bradford assay. Equal amounts of protein were separated by SDS-PAGE. Specific antibody binding against TSPO and ß-actin was detected by chemiluminescence. **a** Densitometrical analysis of TSPO expression of at least 6 independent experiments. Data are presented as mean ± SEM % of control (cells treated with solvent). **b** One representative experiment is shown. Ctrl: control.

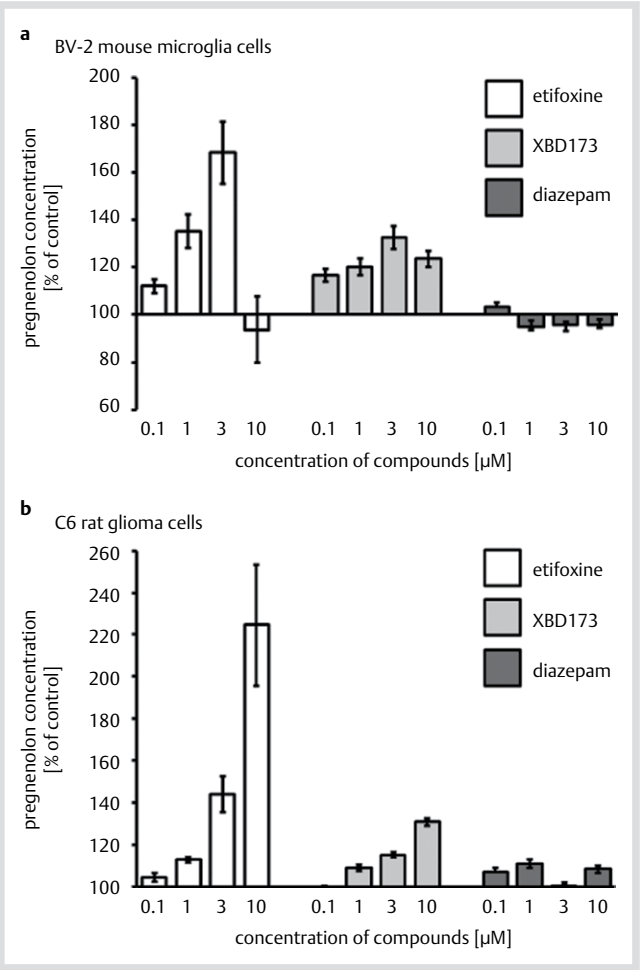

Fig. 4 Enhancement of neurosteroid synthesis. Pregnenolone concentrations after 24 h incubation with XBD173, etifoxine or diazepam. Cells were treated with the respective compounds at different concentrations: 0.1 µM, 1 µM, 3 µM and 10 µM. Trilostane was added to the assay buffer to prevent further metabolization of pregnenolone. Supernatants were used in an enzyme-linked immunosorbent assay to quantify pregnenolone levels. Pregnenolone concentrations are presented in percent of control (cells treated with solvent; 100 %) of at least 6 independent experiments. **a** BV-2 mouse microglia cells. **b** C6 rat glioma cells.

Discussion

The aim of this study was to characterize the pharmacological properties of different anxiolytic compounds with regard to neurosteroid synthesis in BV-2 and C6 glioma cells. More precisely, we investigated TSPO binding affinity, TSPO protein expression and pregnenolone synthesis induction after treatment with the TSPO ligands XBD173 and etifoxine compared to the benzodiazepine diazepam.

XBD173, etifoxine and diazepam all showed considerable binding affinities to TSPO in [^{3}H]PK11195 radioligand binding assays in both cell lines under investigation (rat astrocytes and mouse microglia). XBD173 turned out to be the ligand with the highest binding affinity, followed by diazepam and etifoxine. Treatment of cells with XBD173, etifoxine or diazepam led to increased TSPO expression in both cell lines already at low concentrations, but without showing a clear concentration-dependence. Pregnenolone synthesis was significantly increased by XBD173 and etifoxine, but only marginally by diazepam.

It appeared surprising that in our study, XBD173 showed a rather weak activation of neurosteroid synthesis in our study in view of the pronounced enhancement of allopregnanolone levels in rat brain after XBD173 treatment compared to diazepam, as shown by Rupprecht and colleagues [10]. However, the evaluation of neurosteroid synthesis measured by pregnenolone levels is limited because pregnenolone needs further metabolization until anxiolytic neurosteroids such as allopregnanolone get synthesized. Putative effects of XBD173 on steroidogenic enzymes involved in the synthesis of allopregnanolone therefore remain to be elucidated [15]. Furthermore, there is a discrepancy between the high binding affinity of XBD173 to TSPO and the rather weak enhancement of neurosteroid synthesis compared to the other compounds. These results indicate that the binding affinity not necessarily determines the pharmacological potency

of a drug. Moreover, different binding affinity patterns for TSPO ligands as found in postmortem brains and in platelets from healthy volunteers may explain variations in therapeutic efficacy [16]. Considering the pharmacological effect of a compound, also changes in the expression of a target protein have to be taken into account. Upregulation of the available amount of a drug target can sometimes be a mechanism underlying the pharmacological effect mediated by this target, in this case increased synthesis of neurosteroids. In case of XBD173 treatment, we found a marked upregulation of TSPO protein expression. However, this effect showed no comparable concentration-dependence as did neurosteroid synthesis. Therefore, TSPO protein upregulation does probably not play an outstanding role for the modulation of neurosteroid synthesis by XBD173. Comparable results in terms of TSPO protein expression were obtained for etifoxine and diazepam, thereby pointing out that TSPO upregulation might rather be an unspecific epiphenomenon upon many different stimuli [17,18].

Also for etifoxine we did not find a correlation between TSPO binding affinity and neurosteroid synthesis. Surprisingly, etifoxine turned out to be the most efficient compound in enhancing neurosteroid synthesis, although it was the TSPO ligand with the lowest binding affinity as revealed by [^{3}H]PK11195 radioligand binding assays. Also the results obtained for etifoxine indicate that TSPO binding affinity seems not to be a good predictor for the capability of a TSPO ligand to enhance neurosteroid synthesis. Considering clinical efficacy, behavioural studies in animals as well as in humans demonstrated potent anxiolytic effects of etifoxine, thereby confirming the crucial role of neurosteroid enhancement such as allopregnanolone in the treatment of anxiety disorders [19]. However, etifoxine can also induce a certain sedation, which might be attributed to the direct modulation of $GABA_A$ receptors.

Diazepam showed an intermediate binding affinity to TSPO, which is surprising in view of previous studies indicating a rather low binding affinity of diazepam to TSPO [3,20]. One possible explanation for such varying binding affinities of the same compound might be different tissue characteristics [21]. However, neurosteroid synthesis was only marginally increased in both cell lines under investigation. Again for this compound we could demonstrate a discrepancy between a proper binding affinity to the drug target and a low pharmacological output in terms of neurosteroid synthesis. These results confirm that the anxiolytic potency of diazepam is rather mediated by its direct modulation of $GABA_A$ receptor function. As previously shown by Kita et al., anxiolytic effects of diazepam in rats were not diminished by trilostane and finasteride, 2 inhibitors of steroidogenic enzymes [22]. However, higher concentrations might have revealed stronger effects on pregnenolone synthesis. Regarding the applied concentrations of compounds an extended range of concentrations that are reached in the cerebrospinal fluid in humans for diazepam was chosen [12]. With regard to side effects, diazepam is a sedative drug. Nonetheless, benzodiazepines are still indispensable in the therapy of anxiety disorders, not least due to their broad therapeutic window.

As has been demonstrated for all compounds, there is a discrepancy between TSPO ligand binding affinity and the enhancement of neurosteroid synthesis as a functional read out. Therefore a certain TSPO binding affinity – as measured by [^{3}H]PK1195 radio ligand displacement – does not necessarily allow conclusions as to the pharmacological efficacy of a compound with regard to induction of neurosteroid synthesis.

Conflict of Interest

The authors declare no conflict of interest.

References

1 *World Health Organization*. Disease and injury regional estimates for 2004. Spreadsheet: DALYs (000s) by cause, countries grouped by income and WHO region (a), estimates for 2004, 2004
2 *Nothdurfter C*, *Rupprecht R*, *Rammes G*. Recent developments in potential anxiolytic agents targeting GABAA/BzR complex or the translocator protein (18kDa) (TSPO). Curr Top Med Chem 2012; 12: 360–370
3 *Rupprecht R*, *Papadopoulos V*, *Rammes G et al.* Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. Nat Rev Drug Discov 2010; 9: 971–988
4 *Papadopoulos V*, *Baraldi M*, *Guilarte TR et al.* Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol Sci 2006; 27: 402–409
5 *Casellas P*, *Galiegue S*, *Basile AS*. Peripheral benzodiazepine receptors and mitochondrial function. Neurochem Int 2002; 40: 475–486
6 *Maeda J*, *Higuchi M*, *Inaji M et al.* Phase-dependent roles of reactive microglia and astrocytes in nervous system injury as delineated by imaging of peripheral benzodiazepine receptor. Brain Res 2007; 1157: 100–111
7 *Ching AS*, *Kuhnast B*, *Damont A et al.* Current paradigm of the 18-kDa translocator protein (TSPO) as a molecular target for PET imaging in neuroinflammation and neurodegenerative diseases. Insights Imaging 2012; 3: 111–119
8 *Nguyen N*, *Fakra E*, *Pradel V et al.* Efficacy of etifoxine compared to lorazepam monotherapy in the treatment of patients with adjustment disorders with anxiety: a double-blind controlled study in general practice. Human Psychopharmacol Clin Exp 2006; 139–149
9 *Schlichter R*, *Rybalchenko V*, *Poisbeau P et al.* Modulation of GABAergic synaptic transmission by the non-benzodiazepine anxiolytic etifoxine. Neuropharmacology 2000; 39: 1523–1535
10 *Rupprecht R*, *Rammes G*, *Eser D et al.* Translocator protein (18 kD) as target for anxiolytics without benzodiazepine-like side effects. Science 2009; 24: 490–493
11 *Boothe DM*. Drugs affecting animal behavior. Veterinary Pharmacology and Therapeutics, Iowa State University Press, ISBN: 08138174398 2001; pp 383–396
12 *Kanto J*, *Kangas L*, *Siirtola T*. Cerebrospinal-fluid concentration of diazepam and its metabolites in man. Acta Pharmacol Toxicol 1975; 36: 328–334
13 *Bradford MM*. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248–254
14 *Da Settimo F*, *Simorini F*, *Taliani S et al.* Anxiolytic-like effects of N,N-dialkyl-2-phenylindol-3-ylglyoxylamides by modulation of translocator protein promoting neurosteroid biosynthesis. J Med Chem 2008; 51: 5798–5806
15 *Nothdurfter C*, *Rammes G*, *Baghai TC et al.* Translocator protein (18 kDa) as a target for novel anxiolytics with a favourable side-effect profile. J Neuroendocrinol 2011; 24: 82–92
16 *Owen DR*, *Gunn RN*, *Rabiner EA et al.* Mixed-affinity binding in humans with 18-kDa translocator protein ligands. J Nucl Med 2011; 52: 24–32
17 *Leschiner S*, *Weizman R*, *Shoukrun R et al.* Tissue-specific regulation of the peripheral benzodiazepine receptor by antidepressants and lithium. Neuropsychobiology 2000; 42: 127–134
18 *Wei XH*, *Wei X*, *Chen FY et al.* The upregulation of translocator protein (18 kDa) promotes recovery from neuropathic pain in rats. J Neurosci 2013; 33: 1540–1551
19 *Ugale RR*, *Sharma AN*, *Kokare DM et al.* Neurosteroid allopregnanolone mediates anxiolytic effect of etifoxine in rats. Brain Res 2007; 1184: 193–201
20 *Papadopoulos VL*, *Baraldi M*, *Guilarte TR et al.* Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol Sci 2006; 27: 402–409
21 *Scarf AM*, *Kassiou M*. The translocator protein. J Nucl Med 2011; 52: 677–680
22 *Kita A*, *Furukawa K*. Involvement of neurosteroids in the anxiolytic-like effects of AC-5216 in mice. Pharmacol Biochem Behav 2008; 89: 171–178