



Supporting Online Material for

Translocator Protein (18 kD) as Target for Anxiolytics Without Benzodiazepine-Like Side Effects

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This PDF file includes:

Materials and Methods
SOM Text
Figs. S1 to S6
Tables S1 to S3
References

Supporting Online Material

Materials and Methods

Supplementary Introduction

Supplementary Data

Supplementary Discussion

Supplementary References

Figures S1-S6

Tables S1-S3

Materials and Methods

Compounds

For preclinical studies, NVP-XBD173 (N-benzyl-N-ethyl-2-(7,8-dihydro-7-methyl-8-oxo-2-phenyl-9H-purin-9-yl) acetamide; AC-5216; molecular weight: 401.46) and alprazolam used in CCK-4 studies were obtained from Novartis, Basel Switzerland, whereas alprazolam used in lactate studies and diazepam, allopregnanolone, flumazenil or PK11195 used for electrophysiological or animal studies were from Sigma (St Quentin Fallavier, France and St Louis, MO, USA), respectively. Chlordiazepoxide was from RBI, MA, USA. Drugs were dissolved in ethanol for electrophysiological studies and prepared as a suspension in Tragacanth gum (0.5% aqueous final solution) (Merck, Darmstadt, Germany) or methylcellulose 0.5% (ANIMED, BioConcept, Switzerland) for behavioural animal studies, respectively. CCK-4 (Sigma, Steinheim, Germany) and sodium lactate (Fluka, Sigma, St Quentin Fallavier, France) were injected in sterile physiological saline. For the human proof of concept study, placebo, XBD173 and alprazolam were supplied by Novartis. XBD173 was administered in 10 mg tablets prepared by Novartis. XBD173, alprazolam and placebo were over-encapsulated to ensure identical appearance for blinding. CCK-4 was obtained from Clinalfa, Läufelfingen, Switzerland.

Animals

Male OF1 mice (OF1/IC, Iffa Credo, Lyon, France) weighing 20-22 g were tested in the elevated plus maze and used for monitoring of food intake. All animals were housed under a 12 h light/dark cycle starting five days before the experiment. Animals had access to water and food ad libitum. For the social exploration test male adult Sprague Dawley rats (OFA/ IC strain, Iffa Credo, Lyon, France) (“residents”) weighing 350–400 g or young Lister Hooded rats (li/ho, Harlan, The Netherlands) (“intruders”) weighing 100–120 g were used. “Intruders” were housed in pairs and the “residents” were individually housed in plastic cages (Macrolon, 42 x 26 x 15 cm) for 2 weeks before the test. All animals lived in the same room and had free access to standard food and water. Neurosteroid measurements were performed in brain homogenates of juvenile male Lister Hooded rats. Lactate-induced panic-like responses were assessed in male Sprague Dawley rats (Centre d’Elevage R. Janvier,

Le Genest-Saint-Isle, France) weighing 290-420 g on the day of stereotaxic surgery. Three to four animals were housed per polypropylene cage (floor area = 900 cm²) under standard conditions. Rats were allowed to habituate for at least 5 days prior to further experiments. After surgery, panic-prone rats were kept isolated for the entire period of experiments, whereas partner rats used for social interaction tests were kept in social groups of 3-4 individuals. CCK-4 induced panic-like responses were investigated in male Lewis rats (Charles River, Sulzfeld, Germany) weighing 170-240 g. These rats were kept five animals per cage under a normal 12h light/dark cycle for 7 days prior to the start of the experiment. Slices for electrophysiological recordings were prepared from brains of male C57BL/6 mice (Charles River, Sulzfeld, Germany). All animal studies were in line with valid animal permissions as issued by the local authorities.

Concentration clamp recordings

WSS-1 cells containing functional GABA_A receptor subunits ($\alpha_1\beta_3\gamma_2$) were used for recordings in a whole cell voltage-clamp configuration as described previously (S1). XBD173 or diazepam was administered via a fast superfusion device at the indicated concentrations. 100 μ M GABA were used because this concentration with an application time of 10 ms yields an effective, but unsaturated activation of postsynaptic GABA_A receptors and still lies within the steep slope of the GABA dose response curve (S2). Thus, the activation of GABA_A receptors can still be potentiated by positive allosteric modulators, e.g. benzodiazepines.

Slice experiments

Anaesthetized male C57BL/6 mice (28–42 days old) were killed by cervical dislocation and the brains were rapidly removed into ice-cold ACSF (artificial cerebrospinal fluid) containing (in mM): NaCl 125, KCl 2.5, NaHCO₃ 25, CaCl₂ 2, MgCl₂ 2, D-glucose 25, and NaH₂PO₄ 1.25 (all from RBI/Sigma, Deisenhofen, Germany). Saturation with a mixture of 95% O₂ / 5% CO₂ (carbogen gas) led to a pH of 7.4. Sagittal slices of the medial prefrontal cortex (mpfC) from male C57BL/6 mice were used for recordings of isolated GABA_A receptor mediated IPSCs (GABA_A-IPSCs) and action potential independent miniature IPSCs (mIPSCs). 350 μ m thick slices were prepared using a microtome (HM 650 V, Microm International, Walldorf, Germany). After incubation in a holding chamber with ACSF (22-25 °C) for at least 60 min, the slices were placed in a submerged recording chamber and superfused with ACSF at a flow rate of 1.5 ml/min (S3). Infrared-phase contrast-enhanced videomicroscopy (Zeiss, Oberkochen, Germany) (S4) was used to visualize the somata of principal neurons within the mpfC. IPSCs in layer II/III neurons of the mpfC were evoked by stimulation with an ultra fine bipolar electrode (12 μ m; FHC, Maine, USA) which was positioned in layer II/III close to the apical dendrite of the recorded neuron. Isolated GABA_A-IPSCs and mIPSCs were measured in the presence of 50 μ M (D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5), 5 μ M NBQX and 200 μ M 3-aminopropyl (diethoxymethyl)phosphonic acid (CGP 35348). For recording of IPSCs cells were held

at -40 mV for 400 ms. At the end of each experiment, 50 μ M picrotoxin were added to the bath solution to verify that all responses are mediated through GABA_A receptors. Series resistance was monitored continuously and compensated in bridge mode. Neuronal input resistance was monitored by injecting hyperpolarizing current pulses (100 ms, -10 mV, 0.5 Hz) through the patch electrode. If input resistance or series resistance showed an alteration of more than 15% during the experiment, the data of the respective neurons were discarded. The liquid junction potential was 7 mV.

The patch pipettes were pulled from thin-walled borosilicate glass tubes with inner filament (outer diameter 1.5 mm, inner diameter 1.17 mm, GC150TF-10, Clark Electromedical Instruments, Pangbourne Reading, UK) and heat polished using a two-step horizontal puller (DMZ-Universal Puller, Zeitz-Instruments, Munich, Germany). Pipettes had a series resistance of 4–6 M Ω , when filled with a solution containing (in mM): K-D-gluconate 130, KCl 5, Mg-ATP 4, Na₂-phosphocreatine 5, HEPES 10, lidocaine-N-ethyl chloride 5; pH 7.4 adjusted with KOH (osmolarity: 305 mOsm). All compounds were from Sigma/Aldrich, Germany. Currents were recorded with a switched voltage-clamp amplifier (SEC 10L, NPI electronic, Tamm, Germany) with switching frequencies of 60–80 kHz (25% duty cycle). All patch-clamp experiments were performed at room temperature (22–25°C). GABA_A-IPSCs were induced by square pulse stimuli (2–5 V, 50 μ s) at a stimulation frequency of 0.5 Hz. To avoid any changes in synaptic response associated with changes in stimulus frequency, once stimulation was commenced at 0.5 Hz, it was maintained at that frequency throughout the duration of the recording. Afferents were initially stimulated at an intensity sufficient to evoke a clear GABA_A-IPSC (at -40 mV) and then stimulus intensity was decreased to a point to evoke all or none responses. All recordings were amplified, filtered (3 kHz) and digitized (9 kHz). The digitized data were stored to a disk on a Power Macintosh G4 computer by a data acquisition and evaluation program (Pulse v. 8.5, HEKA Electronic GmbH, Lambrecht, Germany).

The amplitudes, frequencies, charge and decay of mIPSCs were studied by continuous recording over 300 s in the presence of 1 μ M tetrodotoxin (TTX). The peak amplitudes and kinetics of the mIPSCs were measured off-line automatically using a mini analysis program with an adjustable amplitude threshold (version 6.0.3. Synaptosoft Inc., New Jersey, USA). Frequencies were calculated by dividing the total number of mIPSC events by the total time sampled. For recording of mIPSCs the pipettes were filled with a solution containing (in mM): Mg-ATP 2, CsCH₃SO₃ 100, CsCl 60, EGTA 0.2, HEPES 10, MgCl₂ 1, lidocaine-N-ethyl chloride 5 and Na₃GTP 0.3 (pH 7.3). Under these conditions the reversal potential for Cl⁻ ions was -28 mV. Cells were clamped at -70 mV.

Elevated plus maze test

The elevated plus-maze apparatus consisted of two open arms (30 x 5 cm) and two enclosed arms (30 x 5 cm, walls 15 cm high) which extended from a common central platform (5 x 5 cm). The configuration formed the shape of a plus-sign with comparable arms arranged opposite one another.

The apparatus was elevated 60 cm above the floor level. The mouse was placed onto the central platform facing an enclosed arm. During a 6 min trial the number of open and closed arm entries, time spent on open arms, and latency to leave the first arm was scored by an observer blind to the respective treatment. Animals from different treatment groups were alternatively tested with trials done during the first half of the light phase. Oral drug treatments were always given 60 min prior to the trial.

Social exploration test

In the social exploration test an “intruder” rat was placed into the home cage of a resident animal. All observations were made in the home cage of the “resident” the floor of which was covered with sawdust. Pairs consisting of one “intruder” animal and one “resident” animal were assigned at random to one of the experimental or the vehicle-treated control groups. In each pair only the “intruder” was treated either acutely or repeatedly for 4 consecutive days twice daily with the last administration on day 5 (test day). One hour after a single or the last of the repeated doses the animal was placed into the home cage of a “resident” animal. The duration of active approach behaviors (sniffing, anogenital exploration, nosing, grooming, licking, playing) of the “intruder” rat was scored manually and cumulatively recorded over a period of 5 min by an experienced observer. Tests were run during the light phase (7 a.m. to 3 p.m).

Assessment of lactate induced panic-like responses in panic-prone rats

Prior to the assessment of lactate induced panic-like responses rats underwent stereotaxic surgery implanting a stainless steel injection cannula and a bicuculline-sensitive reactive site within the dorsomedial hypothalamus was determined by injection of a bicuculline methiodide solution. Then, the GABA synthesis inhibitor L-allylglycine (14 nmoles/ μ l) was delivered via an Alzet osmotic mini-pump (0.25 μ l/h) into this region for 4 weeks thereby rendering rats chronically anxious and more susceptible to panic-like responses following a lactate infusion (panic prone rats) (S5). After at least 3 days recovery rats were equipped with an arterial and a venous catheter for blood pressure recordings and lactate infusion, respectively. On postoperative days 7 to 22 rats were challenged intravenously with a 1 M lactate solution (infusion of 10 ml/kg, 0.2 ml/min) or saline and immediately tested for social interaction. For this purpose, an experimental panic-prone animal was placed into an open field (90 x 90 x 40 cm) together with an unknown partner. Both animals were allowed to freely interact for a period of 5 min. In addition to the social interaction time, locomotor activity was monitored by recording the number of crossed lines in the test arena. XBD173 or alprazolam were administered 60 min prior to the lactate challenge.

Assessment of CCK-4 induced panic-like responses in rats

Rats were group-housed with at least three animals per cage until 15 min prior to the experiment. 2 min following s.c. injection of 10 mg/kg CCK-4 rats were placed with their forepaws on a 7 cm high

wooden block and the time remaining in a frozen position, i.e. until moving down from the wooden block, was recorded for at least five times with 2 min intervals. Only those rats showing freezing for at least 40 s during a screening experiment, which preceded the experiment evaluating the anxiolytic potential of the respective compounds by a one week, were used. XBD173 was administered orally 60 min and alprazolam was injected 15 min prior to the CCK-4 challenge, respectively. Experiments were performed in a dimly lit room between 8 am and 12 am.

Analytical methods

Concentrations of allopregnanolone (5 α -pregnan-3 α -ol-20-one) in rat cortex homogenates were quantified following extraction with ethyl acetate, purification with high performance liquid chromatography (HPLC) and derivatization with heptafluorobutyric acid anhydride (HFBA) by means of gas-chromatography/electron-impact mass spectrometry (MS) using deuterium-labelled allopregnanolone as internal standard as described previously (S6). The lower detection limit was 1 pg. XBD173 was analyzed by a validated HPLC-MS/MS assay with an assay imprecision (bias) of $16.9 \pm 7.7 \%$, $3.5 \pm 1.0 \%$ and $4.0 \pm 4.5 \%$ (mean \pm SD) as determined in plasma control samples containing 0.598, 40.0 and 400 ng/mL, respectively. Alprazolam and α -hydroxyalprazolam were also analyzed by a validated HPLC-MS/MS assay with an assay imprecision (bias) of $11.2 \pm 1.9 \%$, $5.2 \pm 5.8 \%$ and $4.6 \pm 1.9 \%$ for alprazolam, and of $9.5 \pm 3.2 \%$, $3.8 \pm 2.7 \%$ and $5.1 \pm 2.7 \%$ (mean \pm SD) for α -hydroxyalprazolam in 0.1, 5.0 and 40.0 ng/mL plasma control samples, respectively. Limits of quantification were 0.2 ng/ml for XBD173 and 0.05 ng/ml for alprazolam and α -hydroxyalprazolam.

Clinical proof of concept trial

Subjects and Study design

A total of 121 healthy male volunteers were recruited using advertisement and from the database of a clinical research organisation. Initially, all subjects were screened to be medication-free and medically healthy by means of medical history, physical examination, electrocardiogram, electroencephalogram and routine laboratory screening including urine drug screening. 100 subjects met these inclusion criteria and were thereafter screened by an experienced psychiatrist with the aid of a structured Mini International Neuropsychiatric Interview (MINI, German version). Based on this second screen 7 subjects had to be excluded due to psychiatric disorders and another 7 subjects due to communication problems or deviation from the normal day-night rhythm. The final study population consisted of 85 subjects with a mean age of 33 ± 6.6 years (mean \pm SD) who participated in the first CCK-4 challenge. One subject was screened as a potential back up subject but did not enter the study. Subjects were challenged with 50 μ g CCK-4 administered intravenously as a bolus injection at 10 a.m. from an adjacent laboratory on day -1. The Acute Panic Inventory (API) was used for psychometric assessment at -5 (baseline) 5, 10, 20, 30 and 60 min after CCK-4 injection as described previously (S7, S8). For

the quantification of the panicogenic effects caused by CCK-4 the area under the time effect curve of the API score (API-AUC) was determined by the trapezoid rule in relation to the challenge time. Only those subjects reacting with a sufficient anxiety response, i.e. showing an increase in the API score > 14 in this initial CCK-4 challenge ($n = 71$), were randomized to one of the five treatment arms (fig. S5). Seven subjects rated with a baseline API score > 14 equivalent to a panic attack already prior to the first CCK-4 challenge were excluded from the exploratory analysis of treatment effects on CCK-4-induced anxiety. Seventy-one subjects were orally treated with either XBD173 (10 mg/day, 30 mg/day, 90 mg/day administered once daily at 8 a.m. after an overnight fast), or alprazolam (1 mg administered twice daily at 8 a.m. and 8 p.m.) or placebo for 7 days under strictly controlled conditions. Seventy subjects completed the seven day drug treatment and then underwent a second CCK-4 challenge on day 7. Randomization was performed by Novartis Drug Supply Management using a validated system. Thereafter, the randomization scheme was locked until completion of the study. Each subject received the identical number of capsules containing medication and/or placebo both in the morning and in the evening to keep the study blind. During the study blood samples were drawn as appropriate to generate pharmacokinetic profiles. Daily physical examinations and records of side effects using the Medical Dictionary for Regulatory Activities (MedDRA) were obtained throughout the entire study. Moreover, withdrawal symptoms were recorded from 12 up to 48 hours after the last dosing. The study protocol was approved by the ethical committee of the Ludwig Maximilian University Munich.

Statistics

Electrophysiological data were analyzed by the t-test for paired samples. Animal data were analyzed by appropriate ANOVAs. When having reached the statistical level of significance ($P < 0.05$), these were followed by appropriate post hoc tests, e.g. Dunnett's test, Student's test or Mann-Whitney U test. For the exploratory human proof of concept study power calculation was performed based on previous data on maximal API scores, which had compared the effects of alprazolam and placebo in the CCK-4 paradigm (S8) allowing for a total one-sided alpha-level of 21 % due to the multiplicity of the three dose level comparisons. Statistical analysis of the effects of XBD173 and alprazolam on CCK-4-induced anxiety (delta between API-AUC on day 7 and at baseline) relative to the placebo-treated group was performed using ANCOVA with treatment group as a fixed effect and baseline API-AUC as a covariate.

Supplementary Introduction

In patients suffering from panic disorder increased plasma concentrations of 3α -reduced neurosteroids, e.g. allopregnanolone, have been reported in the absence of panic attacks (S9, S10), whereas during panic attacks induced either by CCK-4 or lactate the concentrations of these steroids were reduced (S11). So far, no data are available on the expression of neurosteroidogenic enzymes in anxiety disorder patients. However, in the mouse model of protracted social isolation there is a reduced

expression of the 5 α -reductase in selected neurons of anxiety related brain areas, e.g. medial prefrontal cortex, hippocampus and basolateral amygdala, together with a reduction in allopregnanolone levels (S12, S13). This mouse model exhibits anxiety related behaviour, enhanced contextual fear as well as impaired fear extinction and may be considered as an animal model for posttraumatic stress disorder (PTSD) (S13).

Supplementary Data

Assessment of food intake before and after withdrawal from chronic treatment with XBD173 and chlordiazepoxide

Male OF1 mice were housed as diads and were orally treated twice daily with either XBD173 (0.3 mg/kg, 3 mg/kg, 30 mg/kg) or chlordiazepoxide (45 mg/kg) for 20 days (n = 15 diads per group). Thereafter, vehicle was administered twice daily during the withdrawal phase for 5 additional days. Food intake was determined as the difference in food weight at the beginning and the end of the respective test day. Cessation of chronic treatment with chlordiazepoxide resulted in a significant decrease in food intake from 12.7 ± 0.3 g/24 h/diad of mice at the end of chronic treatment to 11.0 ± 0.3 g/24 h/diad of mice on day 1 after withdrawal (mean \pm SEM, $P < 0.05$, Mann-Whitney U test) as described previously (S14), which was no more significant on subsequent withdrawal days. In contrast, treatment with three different doses of XBD173 did not reduce food intake on either withdrawal day. The highest dose of XBD173 studied (30 mg/kg) exceeded that required for anxiolytic activity (0.1 mg/kg) by 300 fold.

Supplementary Discussion

Because only the 90 mg dose of XBD173 showed statistically significant superiority over placebo with regard to the attenuation of CCK-4 induced anxiety, further clinical studies should include doses above that of 90 mg to determine the optimal dose range for the anxiolytic efficacy of XBD173 under clinical conditions. It has been shown that also etifoxine targets the translocator protein (18 kDa) (S15) and may exert anxiolytic effects in patients suffering from adjustment disorders with anxiety (S16). In contrast to XBD173, etifoxine has direct potentiating effects on GABA_A receptors (S17). Thus, no firm conclusion can be drawn whether the putative anxiolytic properties of etifoxine are related to its direct effect on GABA_A receptors or its effects on the translocator protein (18 kDa). This is further supported by animal data showing that the anticonflict effects of etifoxine are only partly antagonized by finasteride, although allopregnanolone levels are drastically reduced (S15). It is further of note that in the clinical etifoxine study one of the side effects of etifoxine is drowsiness (S16), which may be conferred via a direct GABA_A mimetic effect of etifoxine. In contrast to etifoxine, XBD173 does not cause drowsiness in animals and humans.

Both XBD173 and allopregnanolone enhance the charge and prolong the decay of IPSCs and mIPSCs (Fig. 1, fig. S2, table S1A), which is in line with the neurosteroidogenic effect of XBD173. In contrast to XBD173, the effects of diazepam on IPSCs are not impaired by finasteride but completely reversed by flumazenil (fig. S2B-C). These observations together with the lack of effects of an anxiolytic dose of diazepam (0.1 mg/kg) on allopregnanolone levels (fig. S1E) exclude that the effects of diazepam on GABAergic neurotransmission shown in Fig 1 and fig. S2 involve neurosteroidogenesis. This is nevertheless in line with previous findings which showed that a neurosteroidogenic effect of diazepam can be observed after incubation with high concentrations for more than one hour (S18), because in the present study the maximal exposure time of slices to diazepam was only 35 min.

Neurosteroid regulation in anxiety disorders, e. g. panic disorder, appears to be rather complex with differences between the absence and presence of panic attacks (S9, S10, S11, Supplementary Introduction). The enhanced concentrations of 3α -reduced neurosteroids in the absence of panic attacks have been suggested as a counter regulatory mechanism against the occurrence of spontaneous panic attacks (S11). Moreover, it has been shown that the changes in neurosteroid levels following challenge with CCK-4 observed in patients with panic disorder (S11) do not occur in healthy controls (S11, S19) in spite of a similar level of anxiety achieved (S19). Thus, there appears to be also a qualitative difference in neurosteroid regulation between patients suffering from panic disorder and healthy controls following challenge with CCK-4. Moreover, it has to be considered that pharmacologically induced anxiety, e.g. by CCK-4, may be different from innate panic with regard to the underlying pathophysiological mechanisms. Nevertheless, the enhancement of neurosteroidogenesis by targeting the translocator protein (18 kDa) may represent a novel therapeutic strategy which is also related to the pathophysiology of mouse models of anxiety (S12, S13) and panic disorder (S9, S10, S11).

Supplementary Figures

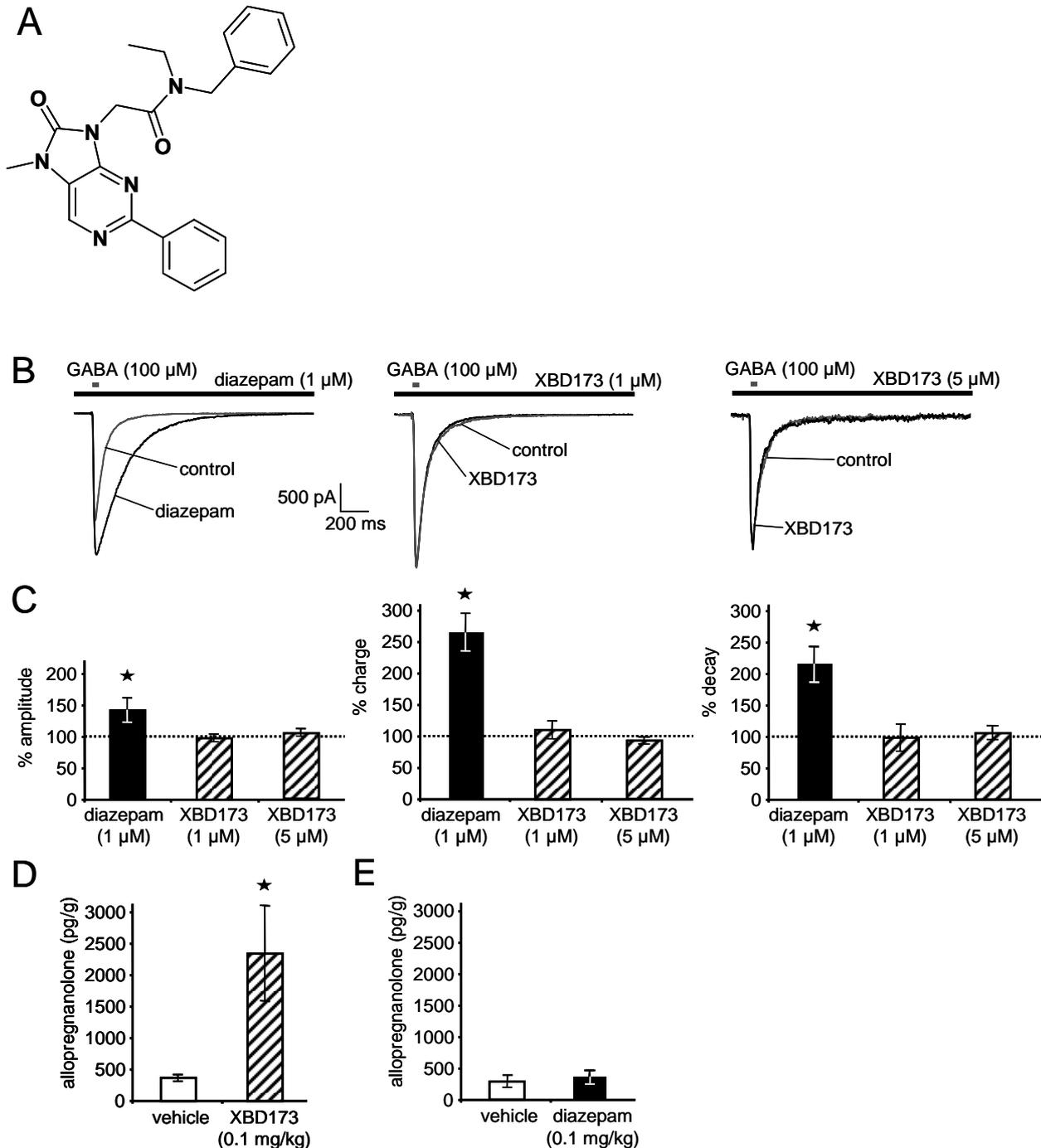


Fig. S1: Effects of XBD173 and diazepam on GABA_A receptor function. **(A)** Chemical structure of XBD173. **(B)** Representative experiments for the modulation of GABA-evoked chloride currents by diazepam or XBD173 in WSS-1 cells expressing $\alpha_1\beta_3\gamma_2$ GABA_A receptor subunits. GABA-evoked currents are shown in the absence and presence of diazepam or XBD173. The upper bars indicate the presence of 100 μ M GABA, the lower bars the presence of 1 μ M diazepam (left panel) and 1 or 5 μ M XBD173 (middle and right panel), respectively. **(C)** Summary of the effects of diazepam or XBD173 on GABA-evoked responses. The bars represent the mean (\pm SEM) of 7 independent experiments. The left diagram shows the effects on the current amplitude, the middle diagram the effects on the charge reflected by the area under the current and the right diagram the effects on the decay time constant (τ) of the current. The responses evoked by GABA alone are set as 100. **(D)** Brain allopregnanolone

content (mean \pm SEM) as determined 1 h following acute oral administration of vehicle or XBD173 (0.1 mg/kg) (n=8 rats per group). (E) Brain allopregnanolone content (mean \pm SEM) as determined 1 h following acute i.p. administration of vehicle or diazepam (0.1 mg/kg) (n=8 rats per group). Electrophysiological data were analyzed by the t-test for paired samples. *P < 0.05, as compared to control experiments. Steroid measurements were analyzed by Kruskal-Wallis one-way ANOVA followed by the Dunn's test. *P < 0.05, as compared to control rats treated with vehicle.

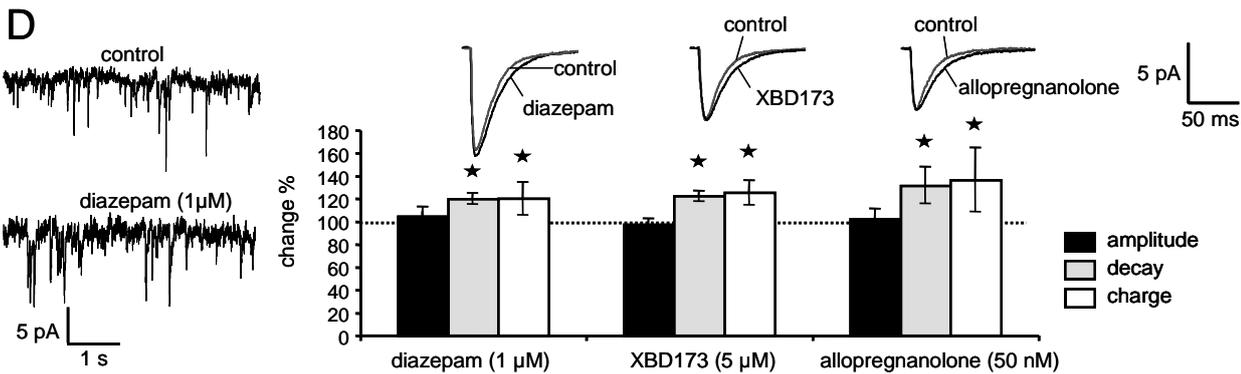
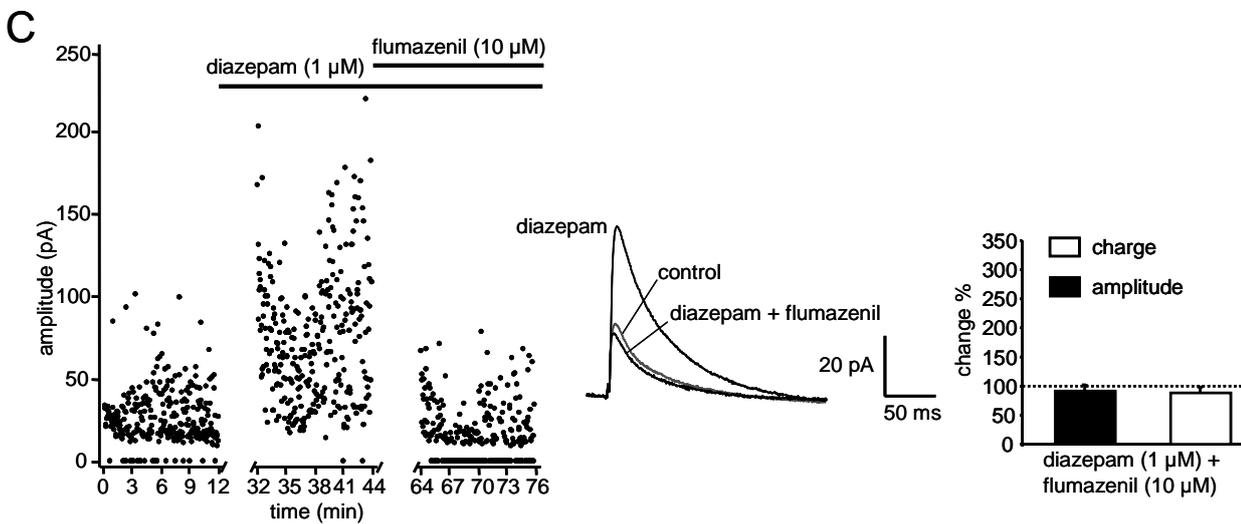
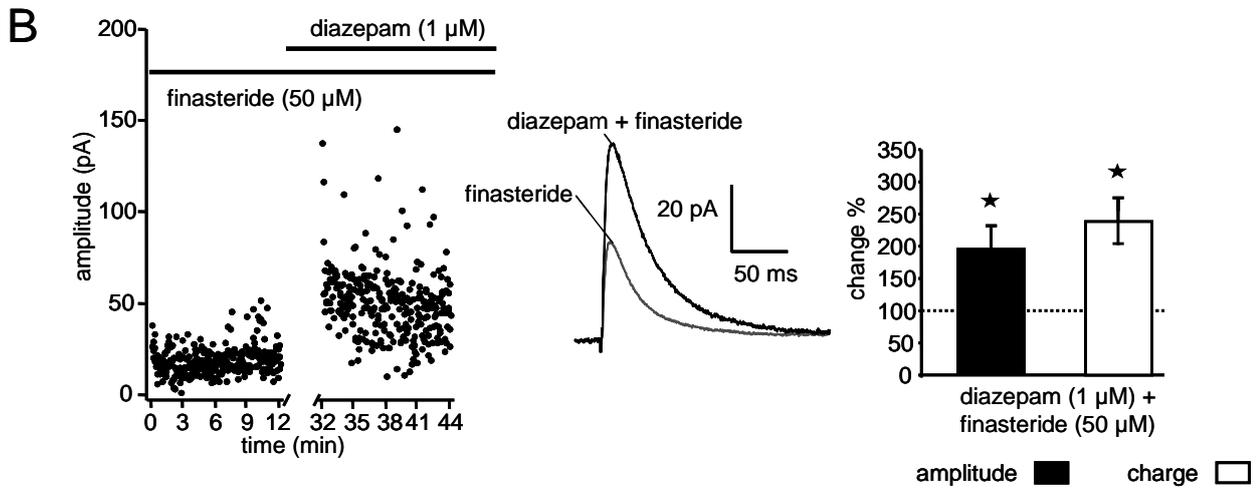
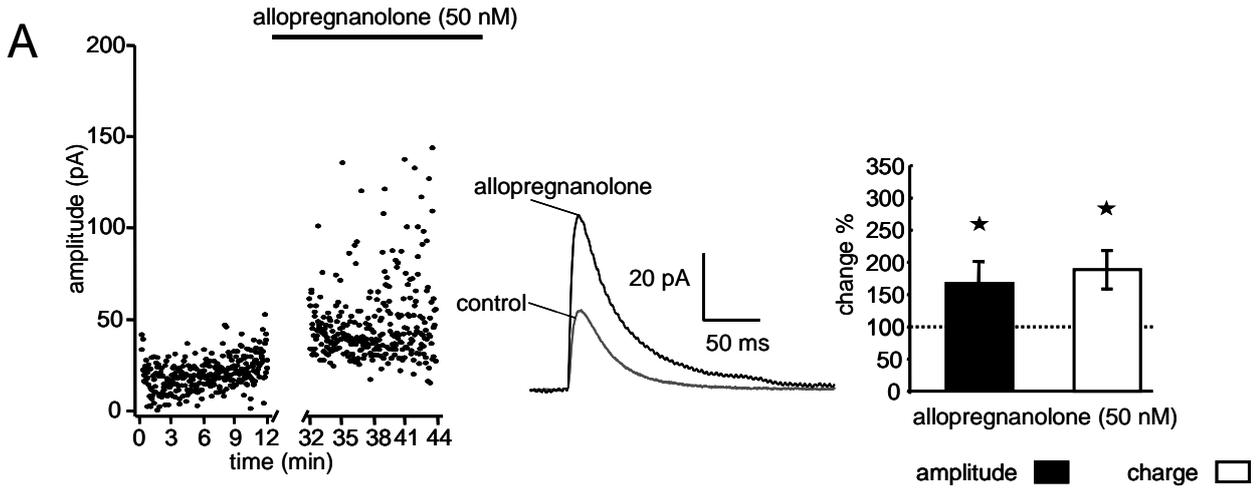


Fig. S2: Effects of allopregnanolone, diazepam and XBD173 on GABAergic neurotransmission in slices of the medial prefrontal cortex. (A-C) Left diagrams: Individual response amplitudes during the course of a representative experiment. Middle diagrams: Averaged traces from all consecutive IPSCs for the control experiments and in the presence of 50 nM allopregnanolone, 50 μ M finasteride and 1 μ M diazepam, or 10 μ M flumazenil and 1 μ M diazepam. Compounds were applied for at least 20 min at the indicated concentrations before IPSCs were recorded. Right diagrams: Averaged data (mean \pm SEM) of all experiments (n = 4-6). (A) Effects of allopregnanolone. The effect of allopregnanolone was monitored with whole cell recordings and minimal stimulation. Allopregnanolone increases the amplitude and charge and prolongs the decay of GABA_A receptor mediated synaptic transmission (table S1A). A potentiation of the amplitude could already be observed at a concentration of 10 nM (table S1B). (B) Modulation of the effects of diazepam by finasteride. Finasteride does not impair the effects of diazepam on amplitude and charge of GABA_A receptor mediated synaptic transmission. (C) Modulation of the effects of diazepam by flumazenil. Flumazenil completely reverses the effects of diazepam on amplitude and charge of GABA_A receptor mediated synaptic transmission. (D) Effects of diazepam, XBD173 and allopregnanolone on amplitude, decay time constant (τ) and charge of action potential independent mIPSCs. Drug treatments did not affect mIPSC frequencies, which excludes presynaptic effects (data not shown). Left diagram: Representative traces of mIPSCs in the absence (control) or presence of 1 μ M diazepam. Right diagram: Superimposed averaged mIPSCs of all detected events (top). Mean (\pm SEM) of all experiments (n = 6) (bottom). The mean amplitude of all recorded mIPSCs in the absence of compounds was 12.1 ± 2.9 pA with a decay time constant (τ) of 23.8 ± 2.9 ms, the mean charge was 0.13 ± 0.05 pC (mean \pm SEM) (n = 18). Diazepam, XBD173 and allopregnanolone increase the charge and prolong the decay of mIPSCs. Electrophysiological data were analyzed by the t-test for paired samples. *P < 0.05, as compared to control experiments.

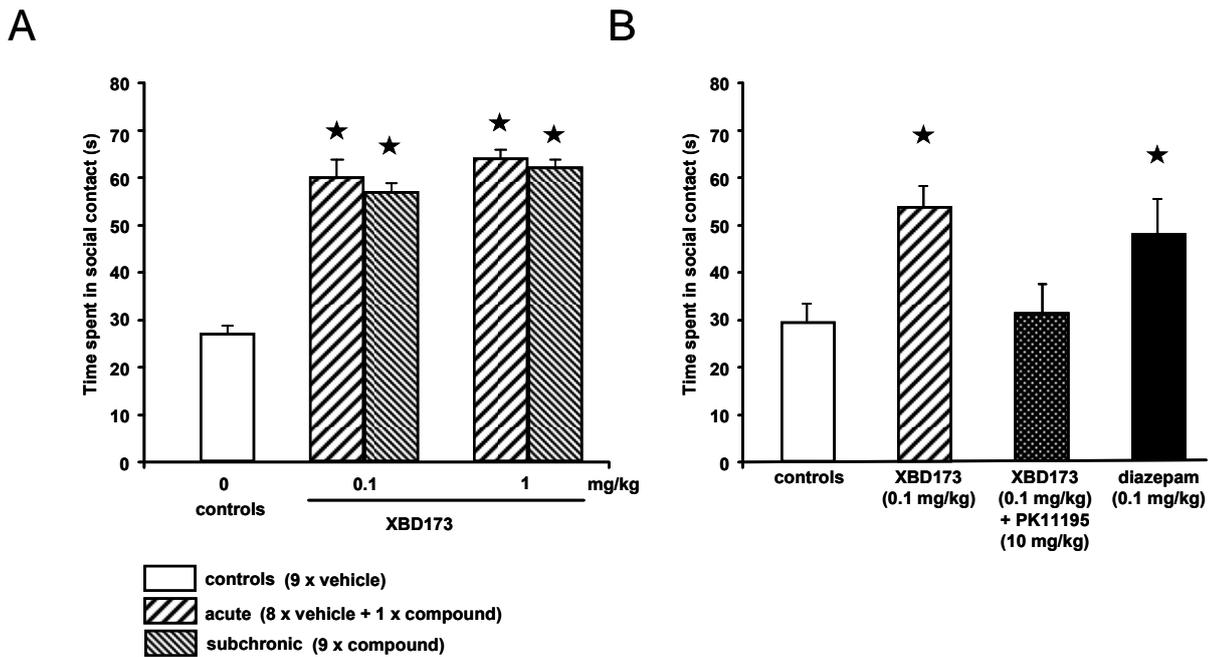


Fig. S3: Effects of XBD173 in the social exploration test in rats. (A) Effect of a single or repeated oral administration of XBD173 on social exploration. Bars represent the mean (\pm SEM) time spent in social contact ($n=12$ rats per group). XBD173 (0.1 or 1 mg/kg) was given orally either as an acute administration or twice daily for 4 consecutive days followed by a last administration on the 5th day. The test was performed one hour after the acute or after the last repeated administration. Control rats received 9 times the vehicle (0.5% Tragacanth). (B) Prevention of the anxiolytic effects of XBD173 by the translocator protein (18 kDa) antagonist PK11195. Bars represent the mean (\pm SEM) time spent in social contact ($n=8$ rats per group). Vehicle, XBD173 (0.1 mg/kg), XBD173 (0.1 mg/kg) in the presence of PK11195 (10 mg/kg), or diazepam (0.1 mg/kg) were given i.p. one hour prior to testing. Data were analyzed by ANOVA followed by Dunnett's test. * $P < 0.05$, statistically significant differences between XBD173 or diazepam treated animals and controls or animals treated with XBD173 in the presence of PK11195.

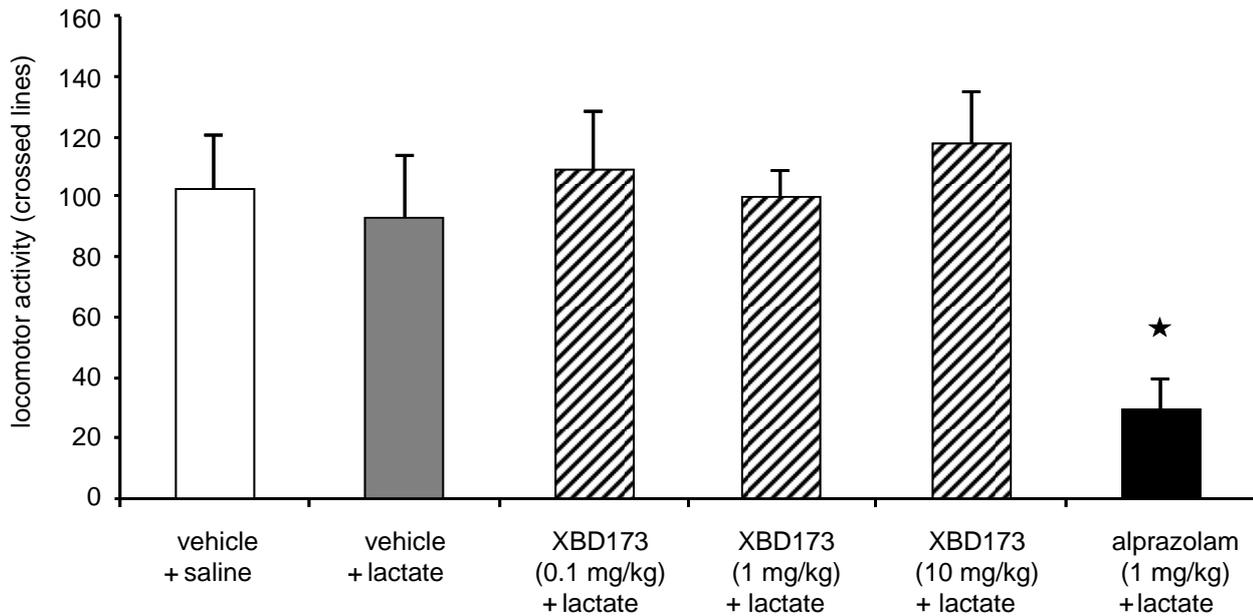


Fig. S4: Effects of acute administration of XBD173 or alprazolam on locomotor activity following infusion of 1 M lactate in panic-prone rats. Bars represent the mean (\pm SEM) locomotor activity (number of crossed lines) during the 5 min open field trial subsequent to lactate administration ($n=8$ rats per group). Panic-prone rats were pretreated (-60 min) with either vehicle, XBD173 (0.1 mg/kg, 1 mg/kg or 10 mg/kg p.o.) or alprazolam (1 mg/kg i.p.) prior to infusion of either saline or a 1 M sodium lactate solution. Data were analyzed by restricted maximum likelihood ANOVA followed by Student's test. * $P < 0.05$, statistically significant difference from vehicle-pretreated control animals following the lactate challenge.

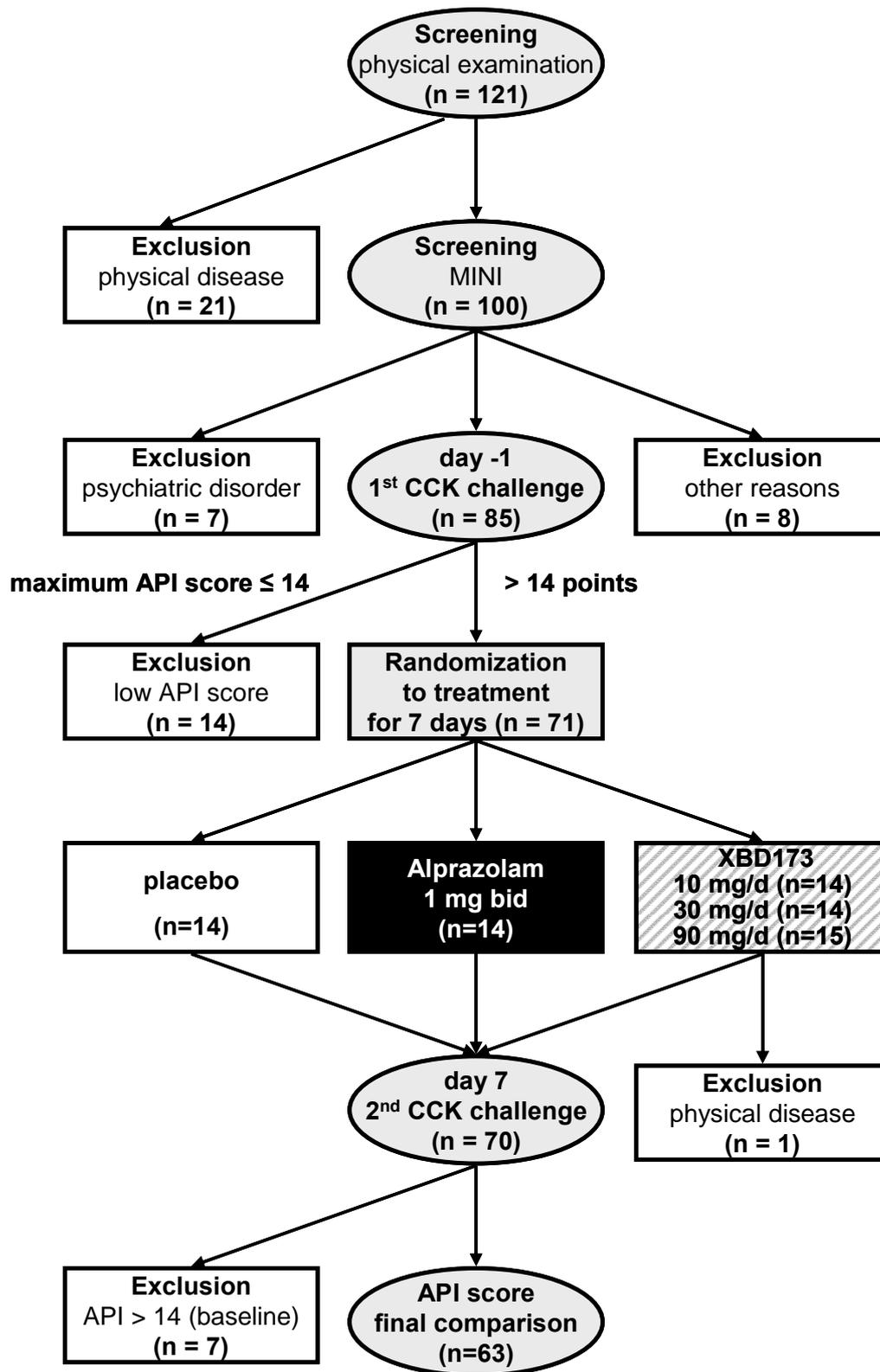


Fig. S5: Study design of the XBD173 proof of concept study. From all eligible subjects ($n = 85$) only those showing a sufficient anxiogenic response to CCK-4 during the first challenge on day -1 as reflected by an increase in the Acute Panic Inventory (API) score of > 14 were randomized to one of the five treatment arms. 71 subjects were randomized, 70 subjects completed the seven day treatment and the second CCK-4 challenge on day 7. One subject treated with 90 mg/day XBD173 did not undergo the second CCK-4 challenge due to a common cold on the challenge day and was therefore replaced by another subject.

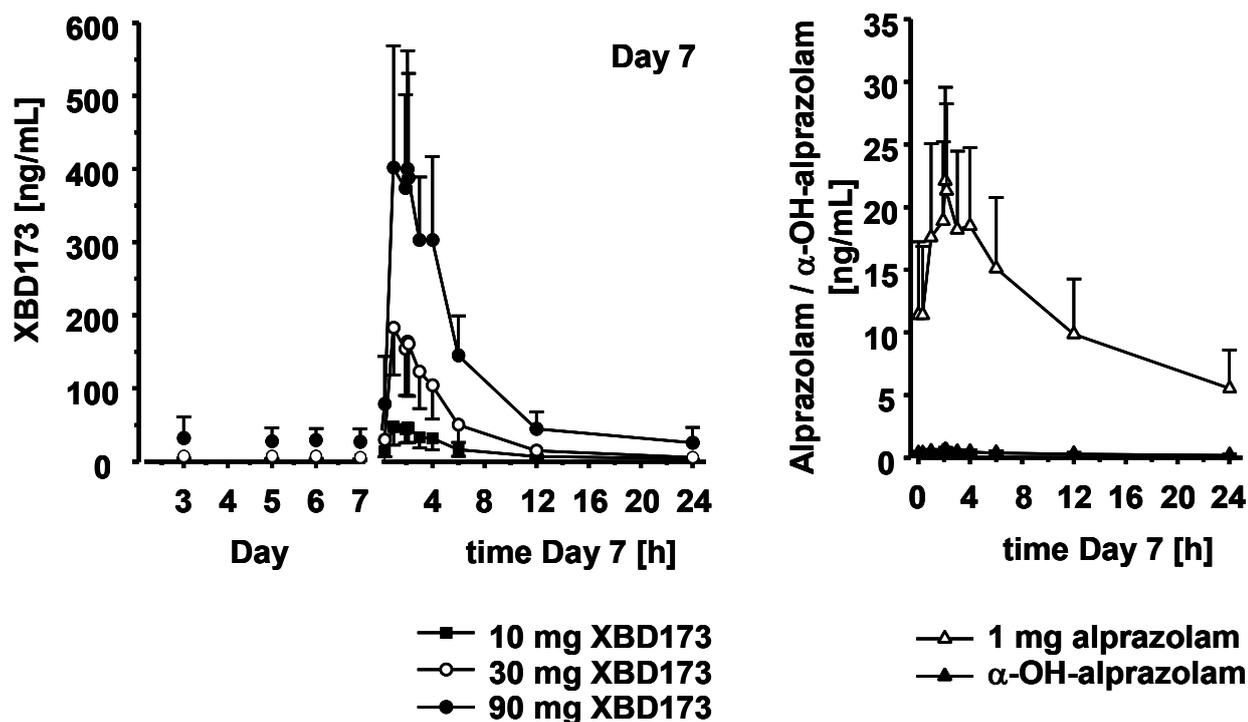


Fig. S6: Pharmacokinetic properties of XBD173 and alprazolam in healthy male volunteers. Arithmetic mean (\pm SD) of XBD173, alprazolam and α -hydroxyalprazolam plasma concentrations after repeated once daily administration of 10, 30 and 90 mg XBD173 or 1 mg alprazolam twice daily ($n = 14$ each). XBD173 plasma concentrations were determined during fasted conditions prior to drug intake. On day 7, plasma concentrations of XBD173, alprazolam and its major metabolite α -hydroxyalprazolam were determined before and after drug intake to assess absorption characteristics after repeated administration. Peak plasma levels of XBD173 are equivalent to low micromolar concentrations.

Supplementary Tables

Table S1: Effects of XBD173, diazepam and allopregnanolone on kinetics of IPSCs

Table S1A: Analysis of the effects of XBD173, allopregnanolone and diazepam on the decay time constant (τ) of IPSCs shown in Fig. 1A-C and fig. S2A-C. Data represent the mean \pm SEM from 4-8 independent experiments. *P < 0.05, statistically significant differences to control experiments. XBD173 and allopregnanolone significantly prolong the decay of IPSCs.

compounds	decay time constant (τ) of IPSCs (% of control)
XBD173 (1 μM)	131 \pm 20 *
XBD173 (5 μM)	132 \pm 15*
XBD173 (1 μM) + finasteride (10 μM)	101 \pm 21
allopregnanolone (50 nM)	133 \pm 8*
diazepam (1 μM)	117 \pm 19
diazepam (5 μM)	116 \pm 20
diazepam (1 μM) + finasteride (50 μM)	120 \pm 23
diazepam (1 μM) + flumazenil (10 μM)	87 \pm 15

Table S1B: Analysis of the effects of 10 nM allopregnanolone on characteristics of IPSCs. Data represent the mean \pm SEM from 6 independent experiments. *P < 0.05, statistically significant differences to control experiments. 10 nM allopregnanolone significantly enhances the amplitude of IPSCs.

	Effects of allopregnanolone (10 nM) on characteristics of IPSCs
amplitude (% of control)	121 \pm 8 *
decay time constant (τ) (% of control)	92 \pm 12
charge (% of control)	111 \pm 15

Table S2: Effects of XBD173 in the elevated plus maze test in mice. Data represent the mean \pm SEM from 16 mice per treatment group. Data were analyzed by Kruskal-Wallis one way ANOVA followed by Dunn's test. * $P < 0.05$, statistically significant differences between XBD173 and vehicle treated animals. XBD173 (0.1 mg/kg) significantly increased the time spent in open arms without affecting activity parameters.

	vehicle	XBD173 (0.03 mg/kg)	XBD173 (0.1 mg/kg)
Ratio (open/total)	0.16 \pm 0.02	0.19 \pm 0.04	0.30 \pm 0.04 *
Time spent on open arms (sec)	21 \pm 4	36 \pm 9	54 \pm 8 *
Total arm entries	14.2 \pm 1.4	15.9 \pm 1.7	15.6 \pm 1.4
Latency to leave the first arm (sec)	50 \pm 4	51 \pm 10	67 \pm 20

Table S3: Side effect profile of XBD173, alprazolam and placebo in healthy male subjects. The Medical Dictionary for Regulatory Activities (MedDRA) was used for classification of adverse events and withdrawal symptoms. Side effects during treatment are shown in table S3A, withdrawal symptoms observed from 12 up to 48 hours after last dosing are depicted in table S3B.

Table S3A

Adverse events (AE) by Body System	XBD173			Alprazolam	placebo
	10 mg/day (N=14)	30 mg/day (N=14)	90 mg/day (N=15)	2 mg/day (N=14)	(N=14)
	n (%)	n (%)	n (%)	n (%)	n (%)
Any Body System (TOTAL)	8 (57.1)	8 (57.1)	8 (53.3)	13 (92.9)	8 (57.1)
Mild	2 (14.3)	3 (21.4)	2 (13.3)	3 (21.4)	5 (35.7)
Moderate	6 (42.9)	4 (28.6)	5 (33.3)	5 (35.7)	3 (21.4)
Severe	0 (0.0)	1 (7.1)	1 (6.7)	5 (35.7)	0 (0.0)
Cardiac disorders	0 (0.0)	0 (0.0)	0 (0.0)	2 (14.3)	0 (0.0)
Eye disorders	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.1)
Gastrointestinal disorders	4 (28.6)	5 (35.7)	4 (26.7)	5 (35.7)	4 (28.6)
General disorders and administration site conditions	6 (42.9)	4 (28.6)	5 (33.3)	11 (78.6)	5 (35.7)
Infections and infestations	0 (0.0)	1 (7.1)	0 (0.0)	0 (0.0)	1 (7.1)
Increase in body temperature	0 (0.0)	0 (0.0)	1 (6.7)	0 (0.0)	0 (0.0)
Musculoskeletal and connective tissue disorders	1 (7.1)	0 (0.0)	2 (13.3)	1 (7.1)	1 (7.1)
Nervous system disorders	7 (50.0)	6 (42.9)	6 (40.0)	11 (78.6)	4 (28.6)
Psychiatric disorders	1 (7.1)	0 (0.0)	1 (6.7)	8 (57.1)	0 (0.0)
Renal and urinary disorders	1 (7.1)	1 (7.1)	1 (6.7)	0 (0.0)	0 (0.0)
Respiratory, thoracic and mediastinal disorders	1 (7.1)	0 (0.0)	2 (13.3)	0 (0.0)	0 (0.0)
Skin and subcutaneous tissue disorders	0 (0.0)	0 (0.0)	0 (0.0)	4 (28.6)	1 (7.1)
Social circumstances	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.1)	0 (0.0)
Vascular disorders	0 (0.0)	0 (0.0)	1 (6.7)	0 (0.0)	0 (0.0)
Total number of AEs	23	30	32	84	21

N = number of subjects per group; n = number of subjects with at least one AE in that category

Subjects with multiple occurrences of an AE are counted only once in the AE category

Subjects with multiple severity ratings for an AE are only counted under the maximum severity

The table includes all adverse events (suspected and not suspected to be related to the study medication)

General disorders: unspecific complaints not related to a specific body system

Administration site conditions: Complaints related to i.v. catheter or infusion

Nervous system disorders: Balance disorder, disturbance in attention, dizziness, headache, memory impairment, sciatica, somnolence

Psychiatric disorders: abnormal dreams, claustrophobia, confusional state, depressed mood, dissociation, fear, indifference, insomnia, listless, paranoia, restlessness, sleep disorders, tension, other psychiatric symptoms

Table S3B

Adverse events defined as withdrawal symptoms (WS) ¹	XBD173			Alprazolam	placebo
	10 mg/day (N=14)	30 mg/day (N=14)	90 mg/day (N=15)	2 mg/day (N=14)	(N=14)
	n (number of subjects)				
Abnormal dreams	0	0	0	4	0
Claustrophobia	0	0	0	1	0
Depressed mood	0	0	0	1	0
Dissociation	0	0	0	1	0
Fear	0	0	0	1	0
Hyperhidrosis	0	0	0	2	0
Insomnia	0	0	0	3	0
Nausea	0	0	0	1	0
Night sweats	0	0	0	1	0
Palpitations	0	0	0	2	0
Paranoia	0	0	0	1	0
Restlessness	1	0	1	4	0
Sleep disorder	0	0	1	5	0
Tension	0	0	0	1	0
Total number of WS	1	0	2	28	0
Total number of subjects with WS (%)	(7%)	(0%)	(6.6%)	(57%)	(0%)

N = number of subjects per group

n = number of subjects with at least one AE in that category

Subjects with multiple occurrences of an AE are counted only once in the AE category

Subjects with multiple severity ratings for an AE are only counted under the maximum severity

¹ the above listed adverse events were defined as withdrawal symptoms if they started later than 12 hours after the last drug administration on Day 7

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