Title page

Characterisation in rats of the anxiolytic potential of ELB139, a new agonist at the benzodiazepine binding site of the $GABA_A$ receptor

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JPET Fast Forward. Published on April 28, 2005 as DOI: 10.1124/jpet.105.084681
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Running title page

Running title:

Anxiolytic effect of ELB139

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Number of text pages: 16

Number of tables: 1

Number of figures: 7

Number of references: 36

Number of words in the abstract: 239

Number of words in the introduction: 739

Number of words in the discussion: 1386

Abbreviation:

ANOVA, analysis of variance; $ATPNa_2$, disodium adenosine triphosphate; b.i.d., twice daily; ED50, 50% effective dose; EGTA, ethylene glycol-bis(2-aminoethyl ether)ethane-N,N,N',N'-tetraacetic acid; ELB139, 1-(4-chlorophenyl)-4-piperidin-1-yl-1,5-dihydro-imidazol-2-on; GABA, γ-amino-n-butyric acid; HEPES, N-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; IC50, 50% inhibiting concentration; NS, not significant; SEM, standard error; SSRI, selective serotonin reuptake inhibitor; TTX, tetrodotoxine; Ω, Ohm;

Recommended section:

Behavioural Pharmacology, Neuropharmacology

JPET Fast Forward. Published on April 28, 2005 as DOI: 10.1124/jpet.105.084681
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Abstract

Benzodiazepines are among the most effective drugs for the treatment of anxiety disorders. However, their use is limited by undesired side-effects including sedation, development of tolerance and drug abuse. The aim of this study was to evaluate the pharmacological profile of ELB139 (1-(4-chlorophenyl)-4-piperidin-1-yl-1,5-dihydro-imidazol-2-on) in different models of anxiety and to correlate these effects with its activity *in vitro*. ELB139 binds with an IC_{50} of 1390 nM to the flunitrazepam binding site in rat forebrain cortical membranes. In rat hippocampal neurones, ELB139 potentiated GABA induced currents without reaching the maximum effect of diazepam indicating a partial benzodiazepine agonism. The potentiation was antagonised by the benzodiazepine antagonist flumazenil. ELB139 (10 and 30 mg/kg p.o.) was active in three different animal models of anxiety, i.e. in the elevated-plus-maze, the light-and-dark box and the Vogel conflict test. The anxiolytic activity in the elevated-plus-maze was almost completely reversed by flumazenil (5 mg/kg i.p.), indicating that interaction with the benzodiazepine binding site is central to the pharmacological activity. No hint of sedation was observed at the doses tested in the three anxiety models and the open field. Also, no development of tolerance was observed within 6 weeks b.i.d. treatment with ELB139 in the elevated-plus-maze test. In summary, ELB139 elicits strong effects on anxiety-related behaviour in rats mediated by its benzodiazepine-like activity without showing sedation or the development of tolerance, a major side-effect of benzodiazepines. These characteristics make the compound a prime candidate for clinical development.

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Introduction

Anxiety disorders are highly prevalent, with an increasing incidence world-wide. The current treatment comprises mainly benzodiazepines and selective serotonin re-uptake inhibitors (SSRI), two classes of drugs which both have the drawback of several side-effects (Pollack, 2002; Shorter and Tyrer, 2003).

Benzodiazepines, still regarded as the most effective drugs for the treatment of anxiety disorders, show a fast onset of the anxiolytic activity. However, they also show undesired side-effects, such as ataxia, sedation, skeletal muscle relaxation, amnesia and, interactions with ethanol and barbiturate. Other major problems are the development of tolerance to therapeutic effects and the potential for drug abuse (Costa and Guidotti, 1996; Dubinsky *et al*., 2002). Several attempts were made to reduce the major side-effects of drugs binding to the benzodiazepine recognition site at the γ-amino-n-butyric acid $(GABA)_A$ receptor, namely sedation and the development of tolerance.

One approach has been made by partial, unselective agonists at the benzodiazepine binding site with high affinity to the $GABA_A$ receptor. Compared with full agonists, imidazenil (for instance) did not induce cognitive deficits, nor was development of tolerance observed after long-term administration in animal models (Auta *et al*., 1995; Costa and Guidotti, 1996). However, the anxiolytic activity of imidazenil found in animals could not be reproduced in man and was not clearly separate from sedation (Atack, 2003). Similar difficulties occurred with abecarnil and bretazenil, partial agonists developed by Schering AG and Roche, respectively (Costa and Guidotti, 1996; Atack, 2003). RWJ-51204, a newer compound shows a separation between anxiolysis and sedation in mice and monkeys but not in rats (Dubinsky *et al*., 2002).

Another approach is to develop agonists that are highly selective for $GABA_A$ receptor subtypes containing α2- and α3-subunits but not α1-subunits, the latter possibly being responsible for sedation (Low *et al*., 2000; Griebel *et al*., 2001). The degree of subtype selectivity seems still to be limited (Griebel *et al*., 2001) but few compounds of that kind have been described. Ocinaplon has shown anxiolytic effects in clinical trials, but the initiation of phase III trials was initially put on hold by the FDA pending the acquisition of further safety data; clinical testing was later recommenced, but at a lower dose than the one tested in phase II (Adis Data Information, 2004). NS2710, developed by

NeuroSearch, showed a sedative effect and affected cognitive function (Atack, 2003). An undisclosed lead compound from the research and development programme of Merck had already reached clinical testing when its development was discontinued for unstated reasons; nevertheless the research programme is continuing (Atack, 2003). A further compound, SL651498, which was found to act as a high-affinity ligand with functional subtype selectivity for α2-containing GABA receptors, has still to prove its ideal profile in human beings (Griebel *et al*. 2001).

A different class of drugs used for the treatment of anxiety are the SSRIs. These drugs are well established as antidepressants and do not induce the major side-effects typical of benzodiazepines, such as tolerance or drug abuse; however, the late onset of their anxiolytic and antidepressive effect limits their therapeutic benefit (Nutt *et al*., 1999). Besides, their therapeutic use is affected by weight gain and sexual dysfunction, which lead patients to discontinue the therapy (Perna *et al*., 2001). A combination of the positive effects of benzodiazepine receptor ligands and SSRIs – i.e., rapid onset of action and potent anxiolytic activity plus the absence of tolerance, abuse potential and sedative potential – could serve as a template for an ideal anxiolytic. However, the search for the ideal anxiolytic is still ongoing.

ELB139 (1-(4-chloro-phenyl)-4-piperidin-1-yl-1,5-dihydro-imidazol-2-on) is a new chemical entity emerging from a research and development programme for anticonvulsants based on *in vivo* screening in co-operation with the NIH (Rostock *et al*., 1998) and on pharmacophore modelling (Figure 1). The present study was initiated to evaluate the anxiolytic activity of the compound as well as to obtain first insight into its possible mechanism of action. The anxiolytic potential was tested in three different animal models. Anxiety-related and locomotor-activity-related parameters were recorded in each test, to obtain more detailed information about the separation between the drug's sedative and anxiolytic effects. To gain initial insight into the mechanism of action of ELB139, its affinity to the benzodiazepine binding site and its effect *in vitro* on GABA-induced current were determined and compared with its effects *in vivo* and their reversibility by the benzodiazepine antagonist flumazenil. In addition, ELB139 was administered sub-acutely and chronically, to assess the risk of tolerance development. Diazepam was used as reference compound.

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Methods

Animals

Male Wistar rats (Crl: (WI) BR, Charles River, Germany) weighing 250–350 g were used. They were housed in groups of five under standard conditions on a 12-h light/dark cycle (light on at 06:00 h) with *ad libitum* access to water and food (ssniff M/R 15, Spezialdiäten GmbH, Soest /Westfalen). The experiments were approved by the Committee on Animal Care and Use of the Federal State of Saxony and carried out following the German Law on the Protection of Animals.

Chemicals

ELB139 and diazepam were obtained from elbion AG (formerly AWD), Germany. Flumazenil was obtained from Tocris, UK. [^{3}H]-flunitrazepam was purchased from Amersham, Germany. For patch-clamp experiments, salts were purchased from Sigma Aldrich, Germany. All other compounds were obtained from Merck Eurolab GmbH, Germany.

Inhibition of specific [^{3}H]-flunitrazepam binding to benzodiazepine binding site

Neuronal membrane fraction from rat forebrain (excluding the cerebellum) were prepared using standard techniques described by Borbe (1985). 150 µl of the membrane fraction was incubated with 0.5 nM [^{3}H]-flunitrazepam and an appropriate concentration of the test compound for 30 min at 4°C. Non-specific binding was determined in the presence of 10 µM diazepam. Binding was terminated by filtration of the incubated membrane fraction using Filtermat A (Pharmacia, Uppsala, Sweden) pre-soaked with 1% polyethylene imine and a Micro Cell Harvester (Skatron, Liver, Norway). Then, the Filtermat A was carefully washed with 0.05 M Tris/HCl-buffer, pH = 7.7, to eliminate unbound radioactivity. The filters were counted in a scintillation counter (Betaplate 1205, Berthold, Wildbad, Germany) to determine the specific binding of [^{3}H]-flunitrazepam. Compounds were screened at 6–10 concentrations to determine IC_{50} and K_i. In the assay, the dissociation constant of [^{3}H]-flunitrazepam was found to be 1.5 nM, its greatest number of binding sites was 0.38 nM and the specific binding was 90%.

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Determination of GABA-induced currents in rat hippocampal neurones

Cell Culture

For GABA-induced current recordings, rat hippocampal neurons were obtained from hippocampal tissue of 18-day-old embryos. Cells were cultivated together with astrocytes, on glass cover slips coated with poly-L-lysine, at a density of 5×10^5 cells/ cm^2. The cells were cultivated in BME supplemented with 10% horse serum, 10% foetal calf serum and glutamine (2 mM). After three days, cytosine-1β–D-arabinofuranoside (5μM) was added in order to inhibit astrocyte propagation and consequent concealment of neurones by large numbers of astrocytes. The cultivation was continued with BME supplemented with 5% horse serum, 5% foetal calf serum and glutamine (2 mM). For the experiments, neurons were used between days 7 and 8 in culture.

Patch-Clamp Recording

The whole-cell variant of the patch-clamp technique was used for the voltage-clamp experiments (Hamill *et al*., 1981). The micropipettes were drawn (equipment from Sutter Instruments Company, Novato, USA) from borosilicate glass capillaries (Science Products, Hofheim, Germany) and heat-polished at the tip (equipment from ALA Scientific Instruments, New York, USA). The pipette resistances were between 2 and 5 MΩ. The composition (mM) of the internal solution was 140 CsCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 N-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) and 11 ethylene glycol-bis(2-aminoethyl ether)ethane-N,N,N',N'-tetraacetic acid (EGTA). The osmolality was adjusted to about 300 mOsmol. The internal solution was prepared in advance and deep-frozen in aliquots of 1 ml. To fill the recording pipettes, the solution was thawed every morning and 2 mM Na_2ATP was added.

Current signals were amplified with an EPC-9 amplifier and were digitised, stored and analysed by using the TIDA system (HEKA Elektronik, Lambrecht Germany). The data were sampled, digitally filtered (Bessel 10 kHz) and stored on a computer disc at a frequency of 2 kHz. For recording, a cover slip was transferred into the recording chamber and permanently superfused with modified

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extracellular solutions containing (mM) 140 NaCl; 5 KCl, 2 CsCl, 1 $MgCl_2$, 10 HEPES, 5 D-glucose, 0.003 Tetrodotoxine (TTX), pH=7.3-7.4 (NaOH).

The cells were clamped at a potential of –80 mV. The concentration of GABA was selected to elicit approximately 20% of the maximum current induced by high concentrations of GABA. This concentration was found to be 3 µM and elicited a current of –268 ± 35 pA (n = 16). The GABA solution or the test compounds (at concentrations between 1 and 100 µM) together with GABA were applied locally onto the clamped cell by using an 8-channel rapid solution exchanger (DAD8; ALA Scientific Instruments, New York, USA). For evaluation of the drug effect, the maximum current amplitude induced by application of 3 µM GABA was set to 100%, and the relative maximum current amplitude induced by the test compound and GABA in relation to the GABA-induced maximum current amplitude in the same cell was calculated.

Animal models of anxiety

Drug administration

ELB139 and diazepam suspended in 0.5% hydroxyethylcellulose were administered orally, respectively 1 h and 30 min before the test. The pre-treatment time was chosen due to previous epilepsy experiments evaluating the time of peak effects (data not shown). For subacute administration the two compounds were administered twice daily for five consecutive days, with the last administrations 1 h and 30 min prior to test, respectively. For long-term exposure, ELB139 (10 and 30 mg/kg p.o.) was administered twice daily on five days per week for six weeks. For antagonism tests, flumazenil was administered at a dose of 5 mg/kg i.p. 20 minutes before the test.

Elevated-plus-maze

The elevated-plus-maze used was a plus-shaped maze made of grey PVC with a black floor. It consisted of two closed arms (14 × 50 cm) with protective walls (27 cm high), two open arms (14 × 50 cm) opposite each other and a central arena of 14 × 14 cm. The maze was elevated 100 cm off the

ground. The apparatus was situated in a separate small shielded square room within the laboratory so that animals were not disturbed by environmental stimuli. The light intensity was 200 ± 50 lux, the light being more intense in the open arms. To start the experiment, the rat was placed in the central arena of the apparatus with the head pointing mid-way between a closed and an open arm. The test lasted for five minutes. Behaviour was videotaped during the session and the following parameters were recorded by the computer programme "VideoMot" (DOS and type II-version, TSE, Bad Homburg, Germany): number of entries into the open arms / total number of entries [%], time spent in the open arms / time spent in all arms [%], and the total number of entries into the arms [n] or, alternatively, the total distance travelled [cm] with the new system as having a higher loading on locomotor activity (Ramos *et al*., 1997).

Light-and-dark box

The apparatus consisted of two chambers (21 × 22 cm) with a grid floor which were connected by a 6.5 × 6 cm opening. One chamber had black walls and the other had white walls. The white chamber was additionally brightly lit. For the experiment, the rat was placed in the white chamber and was allowed to explore the two-chamber area for five minutes. The following parameters were recorded by a laboratory assistant: number of transitions between the two chambers [n] and time spent in the light chamber [sec].

Vogel conflict

A modification of the method of Vogel *et al*. (1971) was used. For the test operant behaviour boxes (30 × 23 × 19 cm) with a stainless-steel grid floor were used (habitest operant cage, Coulbourn instruments, Allentown, USA). A water bottle with a metal drinking-tube was fitted from the outside to the box so that only the drinking-tube extended into the box. This tube was connected to an electric-shock generator (precision regulated animal shocker, Coulbourn instruments, Allentown, USA) which could produce a 0.5-mA shock when the tongue of the rat touched the tube.

The test required three consecutive days in which drinking sessions were performed once a day, always at the same time. On the first day rats were allowed to drink water for 15 minutes without

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being punished, to accustom them to the operant box. They were then put back into their home cage and left without water for 24 hours. On the second day, water was replaced by a 5.0% glucose solution and rats were allowed to drink for five minutes during the training session. They were again left without water for the next 24 hours in their home cage. On the last day, the rats were again offered drinking water. The whole test session lasted for 210 s. For the first 30 s, rats were allowed to drink water without the licks being punished. During the remaining 180 s, rats received a mild electric shock when they touched the drinking tube. The number of unpunished [n] and punished [n] licks were counted by a computer programme "Graphic state notation" (Coulbourn instruments, Allentown, USA).

Open field

The MotiTest Apparatus (TSE, Bad Homburg, Germany) was used for this experiment. The test area consisted of a squared arena (45 × 45 cm) with protective Plexiglas walls (20 cm high) where rats could move freely. Horizontal movements were recorded by 32 infrared photocells arranged along the bottom of each wall of the arena. Vertical movements (rearings) were recorded by a horizontal row of 32 infrared photocells 12 cm above the floor. The light intensity was 150 ± 50 lux. The centre of the arena was defined as the central 50% of the arena by the computer programme. To start the experiment, the animals were placed in the middle of the squared arena and movements were recorded for ten minutes. The following parameters were recorded by the computer programme "ActiMot" (TSE, Bad Homburg, Germany): active time [s], total distance travelled [m], number of rearings [n], distance travelled in the centre / total distance [%] and active time spent in the centre / total activity time [%].

Statistical Analysis

Results are shown as mean ± SEM. The *in vivo* results were analysed by one-way analysis of variance (ANOVA). The Tukey test was used for individual comparison. $P < 0.05$ was regarded as significant.

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Results

Inhibition of specific [^{3}H]-flunitrazepam binding to benzodiazepine binding site

ELB139 inhibited the binding of the specific radioligand [^{3}H]-flunitrazepam to the benzodiazepine receptor with an IC_{50} value of 1390 ± 32.1 nM and a K_i value of 1040 ± 24.1 nM. (mean ± SEM). The IC_{50} and the K_i of diazepam accounted for 9.1 ± 0.7 nM and 6.8 ± 0.6 nM, respectively (mean ± SEM).

GABA-induced currents

ELB139 was tested at the concentration of 1, 10 and 100 µM. Administered onto the neurones in the absence of GABA, ELB139 elicited no current. Administration of GABA alone (3 µM) induced a chloride current of –268 ± 35 pA (*n* = 16) which is approximately 20–30% of the maximum current inducible by high concentrations of GABA. If ELB139 at the above concentrations was administered with GABA (3 µM), the compound enhanced the current concentration-dependently to 108.8 ± 13.3, 162.7 ± 17.5 and 165.9 ± 19.1%, respectively of the value without ELB139. 100% represents the current elicited by administration of 3 µM GABA alone (Figure 2).

To test whether the effect of ELB139 is mediated by the activation of the benzodiazepine binding site, ELB139 was tested together with flumazenil (10 µM). ELB139 was tested at 10 µM, because the maximum potentiation was already reached at this concentration. In these experiments the potentiation by diazepam reached 216 ± 9% and was reduced to 85 ± 5 % by flumazenil. Similarly, 10 µM ELB139 potentiated the effect of GABA to 149 ± 5% and the effect was fully blocked to 94 ± 7% (Figure 3). In direct comparison ELB 139 at the high concentration of 100 µM elicited a potentiation of 173 ± 11 % whereas diazepam tested at the same neurones reached 211 ± 24% (n=5), demonstrating that ELB139 shows only partial agonism at the GABA receptor.

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Animal models of anxiety

Elevated-plus-maze

Acute effect

The results are shown in Figure 4. ELB139 showed an effect on the anxiety-related parameters starting at 10 mg/kg p.o. At 30 mg/kg p.o. ELB139 significantly increased the percentage of entries into open arms ($F(2.82) = 8.15$; $p < 0.001$; $n = 12$) and the percentage time spent in the open arms ($F(2.82) = 4.19$; $p = 0.013$; $n = 12$). Diazepam was effective in the elevated-plus-maze at 6 mg/kg p.o., distinctly raising the percentage of entries into open arms ($F(2.82) = 8.15$; NS; $n = 12$) and significantly increasing the percentage time spent in them ($F(2.82) = 4.19$; $p = 0.033$; $n = 11$). Both compounds slightly increased the total number of entries, being significant against vehicle-treated control with ELB139 at 10 mg/kg p.o. ($F(2.82) = 6.74$; $p = 0.002$ for ELB139 at 10 mg/kg, NS for ELB139 at 30 mg/kg and diazepam at 6 mg/kg; $n = 12$).

Development of tolerance

To evaluate the risk of tolerance development towards the anxiolytic effect, the elevated-plus-maze test was used after repeated administration of both, ELB139 and diazepam (Figure 5). After five consecutive days of b.i.d. administration ELB139 at 30 mg/kg p.o. still significantly increased the two anxiety-related parameters of the elevated-plus-maze, the percentage of entries into open arms ($F(3.81) = 4.24$, $p = 0.39$, $n = 5$) and the percentage time spent in them ($F(3.81) = 6.70$, $p = 0.015$, $n = 5$), whereas diazepam at 4 mg/kg p.o. b.i.d. lost its effect on these two anxiety-related parameters ($F(3.81) = 0.75$ and 0.44, respectively; NS; n=5). There was no effect on the total number of entries for both compounds ($F(3.81) = 0.10$ (ELB139) and 0.60 (diazepam); NS, $n = 5$).

The anxiolytic effect of ELB139 persisted even after 6 weeks of treatment. After 6 weeks of administration ELB139 at 30 mg/kg p.o. significantly increased the percentage of entries into the open arms ($F(3.35) = 4.36$; $p < 0.017$; $n = 10$) and the percentage time spent in them ($F(3.35) = 4.30$; $p < 0.019$; $n = 10$). There was no significant difference between the groups in respect of the total distance travelled ($F(3.35) = 2.73$; NS; $n = 10$). The results are shown in Figure 5.

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Reversal of the acute effect by flumazenil

To assess whether, and to what extent, the low-affinity partial agonistic effect of ELB139 at the benzodiazepine binding site might contribute to the anxiolytic effect, the benzodiazepine antagonist flumazenil was used in a different experiment. The results are shown in Figure 6. At 30 mg/kg p.o. ELB139 again significantly increased the percentage of entries into open arms ($F(3.32) = 4.23$; $p = 0.023$; $n = 10$) and the time spent in them ($F(3.32) = 14.37$; $p < 0.001$; $n = 10$) further confirming the anxiolytic potential of the compound in this model. The total distance travelled was unchanged by ELB139 at this dose ($F(3.32) = 1.99$; NS; $n = 11$). This anxiolytic effect was almost completely reversed by flumazenil at 5 mg/kg i.p.; the percentage entries into the open arms did not differ from vehicle-treated rats ($F(3.32) = 4.23$; $p = 0.780$; $n = 11$). However, the time spent in the open arms remained slightly but significantly increased in comparison with vehicle-treated rats ($F(3.32) = 14.37$; $p = 0.020$; $n = 11$).

Diazepam at 4 mg/kg p.o. also increased the percentage time spent in the open arms significantly ($F(3.32) = 8.51$; $p < 0.001$; $n = 10$) and the percentage entries into them distinctly ($F(3.32) = 2.29$; NS; $n = 10$). The anxiolytic effect of 4 mg/kg p.o. diazepam was also reversed by flumazenil. The entries into the open arms did not differ between ELB139-treated and vehicle-treated rats ($F(3.32) = 2.29$; NS; $n = 11$). The percentage time spent in the open arms also remained slightly increased, but did not reach level of significance ($F(3.32) = 8.51$; $p = 0.155$; $n = 11$). The significant increase of the total distance travelled induced by diazepam at 4 mg/kg p.o. ($F(3.32) = 11.12$; $p < 0.001$; $n = 10$) was totally reversed by flumazenil, ($F(3.32) = 11.12$; NS; $n = 11$).

Light-and-dark box

ELB139 significantly increased the number of transitions between the light and the dark chamber, starting at 10 mg/kg p.o. ($F(2.87) = 3.43$; $p = 0.037$; $n = 10$). Diazepam at 6 mg/kg p.o. significantly increased the number of transitions ($F(2.87) = 3.43$; $p = 0.021$; $n = 10$). The time spent in the light chamber was distinctly increased with diazepam and ELB139 at 6 and 10 mg/kg p.o., respectively ($F(2.87) = 1.97$; NS; $n = 10$).

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Vogel conflict test

Data are shown in Figure 7. ELB139 showed an effect on the number of punished licks starting at 10 mg/kg p.o. It significantly increased the punished licks at 30 mg/kg p.o. ($F(2.83) = 3.38$; $p = 0.043$; $n = 12$) without changing the unpunished licks ($F(2.83) = 3.37$; NS; $n = 12$). Diazepam significantly increased the number of punished licks at 10 mg/kg p.o. ($F(2.83) = 3.38$; $p = 0.041$; $n = 12$). However, the number of unpunished licks were significantly decreased to the control group at this dose, either ($F(2.83) = 3.37$; $p = 0.042$; $n = 12$).

Open field

The data are shown in Table 1. In the open field test ELB139 did not affect the parameters related to locomotor activity, i.e. active time ($F(2.82) = 2.68$; NS; $n = 12$), distance travelled ($F(2.82) = 2.04$; NS; $n = 12$) and number of rearings ($F(2.82) = 1.58$; NS; $n = 12$). The ratio of the distance travelled in the centre of the area to the total distance travelled was distinctly but not significantly increased by ELB139 at 30 mg/kg p.o. ($F(2.82) = 2.17$; NS; $n = 12$). Diazepam at 6 mg/kg p.o. distinctly increased the active time ($F(2.82) = 2.68$; NS; $n = 12$), the total distance travelled ($F(2.82) = 2.04$; NS; $n = 12$), the number of rearings ($F(2.82) = 1.58$; NS; $n = 12$) and the distance travelled in the centre of the area ($F(2.82) = 2.17$; NS; $n = 12$).

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Discussion

The aim of the present study was to evaluate the pharmacological profile of ELB139 in different models of anxiety and to correlate these effects with its activity *in vitro*. The *in vitro* studies showed ELB139 to be a low-affinity, partial agonist at the benzodiazepine binding site. Its IC_{50} for the inhibition of specific [^{3}H]-flunitrazepam binding was about 150 times higher than that of diazepam (Costa and Guidotti, 1996; Nazar *et al*., 1997; Dubinsky *et al*., 2002). Electrophysiological experiments in hippocampal neurones characterise the compound as a partial agonist as the potentiation of GABA-induced currents did not reach the potentiation obtained with diazepam, even after administration of 100 µM, a dose approximately 95-fold higher than the IC_{50}. The potentiation of GABA-induced currents is completely antagonised by flumazenil, further supporting the benzodiazepine binding site interaction.

The anxiolytic activity of ELB139 was evaluated in three animal models of anxiety, two ethological models (elevated-plus-maze and light-and-dark box) and one based on conditioned fear (Vogel conflict). This approach allowed us to test the effect of the compound on a broader spectrum of behavioural facets belonging to different anxiety disorders. It has not yet been tested in the social interaction test, an animal model of social phobia (Rodgers, 1997). However, although the social interaction test is described as detecting another type of anxiety that is differentially modulated by $GABA_A$ receptors, benzodiazepines have been shown to be active in this test, too (Gonzales *et al*., 1998). In all tests used, ELB139 starting at 10 mg/kg p.o. showed an effect on anxiety-related parameters (Pellow *et al*., 1985; Pellow and File, 1986; Kennett *et al*., 2000, Choleris *et al*., 2001). It increased the number of transitions significantly and the time spent in the light chamber distinctly in the light-and-dark box and the percentage of time in the centre of the open field at 10 mg/kg, while in the other anxiety tests significant effects were consistently seen at 30 mg/kg. Diazepam, used as reference compound in all experiments, showed significant effects at 4 to 10 mg/kg p.o. These doses lie in the range described for diazepam in other studies, i.e., between 2 and 10 mg/kg depending on the test and the rat strains used (Wada and Fukuda, 1991).

These data indicate a discrepancy between the low affinity of ELB139 for the benzodiazepine binding site of rat forebrain membranes in combination with the partial agonism and the effective dose in

comparison with diazepam. The 150-fold lower affinity is not reflected in a similar separation of the active *in vivo* doses which were only 3–7-fold higher, depending on the model and the vehicle used. Furthermore, in spite of the partial agonism, ELB139 showed a reproducible and consistent anxiolytic effect at 30 mg/kg, the extent of which was comparable with that of diazepam, as seen with the five elevated-plus-maze runs throughout the year, with 11–12 animals/group (Figures 4–6). This may raise the question of whether other mechanisms may contribute to the potent anxiolytic activity of ELB139. Since the activity of ELB139 could be antagonised with flumazenil to an extent similar to that published for diazepam (Wada and Fukuda, 1991), the benzodiazepine interaction is very likely to be central to the anxiolytic activity. First data, however, indicate that ELB139 may be highly subtype-selective for the α3-subunit of the benzodiazepine binding site. As the α3-subunit is not the major subunit in the hippocampus or the forebrain, both the binding and the electrophysiological experiments may fail to reflect the actual potency of ELB139 on GABA subunits. Studies to evaluate further the subtype selectivity are under way (Rabe *et al*, in press).

A major problem of benzodiazepine-like anxiolytics is that their anxiolytic activity cannot be clearly separated from sedation (File, 1990; Costa and Guidotti, 1996; Dubinsky *et al*., 2002; Atack, 2003). Thus, at high doses diazepam starts to reduce the activity of the rats, as hinted at by the significantly reduced unpunished licks in the Vogel conflict test, a parameter related to locomotor activity (Nazar *et al*., 1997, Kennett *et al*., 2000). To evaluate the safety profile of ELB139, in each test of the present study the parameters predominately related to activity were recorded in parallel to the anxiety-related parameters. We determined the unpunished licks in the Vogel conflict test and the total number of entries and total distance travelled in the elevated-plus-maze test (Ramos *et al*, 1997). In both tests, these parameters were not significantly affected by ELB139 at 30 mg/kg p.o. For the light-and-dark box it is more difficult to separate anxiety- and locomotor activity-related parameters, as the number of transitions between the two chambers is influenced both by anxiety and by activity (Hascoet and Bourin, 1998). The distance travelled in the dark chamber is a more closely related to activity but could not be detected in our system (Hascoet and Bourin, 1998). These results indicate that the anxiolytic effect of ELB139 at 30 mg/kg p.o. is not significantly affected by sedation. Only in the open field at 30 mg/kg p.o. was a slight reduction of the activity to be seen, although the total distance

JPET Fast Forward. Published on April 28, 2005 as DOI: 10.1124/jpet.105.084681
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travelled was unchanged at this dose. The activity comprises active locomotion and static movements, whereas the total distance travelled almost exclusively reflects locomotion. Therefore this reduction of activity seems to be due to a decrease of the static movements rather than to locomotion. This can be supported by data obtained in the RotaRod test and the alcohol interaction test. The impairment of RotaRod performance has an ED_{50} of 265 mg/kg p.o. for ELB139 indicating that motor activity is not significantly affected at 30 mg/kg orally. Likewise, the compound was not found to amplify significantly the depressant effects of ethanol at 100 mg/kg p.o. (Dost, 2004). As static movements comprise different types of activity, such as breathing and grooming, it is difficult to put them down to a certain behavioural response. Sedation would reduce both static and locomotor activity. Interestingly, Blanchard *et al.* (1991) and Homberg *et al.* (2002) have found that enhanced anxiety-related behaviour is to a certain extent accompanied by an increase in grooming behaviour.

Diazepam at lower anxiolytic doses (2-6 mg/kg p.o.) is described as significantly increasing locomotor activity (Dawson *et al.*, 1995). Here, this is predominantly seen in the open field (Table 1). This hyperactivity is discussed differently. Partly, it is associated with an increased exploratory activity due to its anxiolytic effect. However, it is also described as an unspecific hyperlocomotion which, as the anxiety-related parameters are influenced by the locomotor activity of the animals, may further enhance theses parameters and, thus, the anxiolytic activity (Dawson *et al.*, 1995; Soderpalm *et al.*, 1991). Additionally, the hyperlocomotion is discussed as a reflection of the psychostimulant effect of diazepam (Ikemoto, 2004). Such an obvious stimulating effect on locomotor activity was not seen with ELB139.

Further problems of benzodiazepine-like compounds are their potential to induce tolerance towards their therapeutic effect, and physical dependence resulting in withdrawal symptoms after cessation of the therapy (Atack, 2003; Follesa *et al.*, 2001). The tolerance is discussed as being caused by a decrease in $GABA_A$ receptors and also by a rearrangement of the $GABA_A$ receptor subunits (Fernandes *et al.*, 1999; Costa and Guidotti, 1996). This phenomenon is seen mainly with full, but also with partial high-affinity agonists (Atack, 2003; Follesa *et al.*, 2001). An exception among the partial agonists is imidazenil, which retains its anticonvulsant activity even after a prolonged period of administration (Costa and Guidotti, 1996). Developing a partial, low-affinity agonist of the

benzodiazepine binding site was conceived as a new approach to avoid the development of tolerance and physical dependence and the occurrence of withdrawal symptoms. In the present study, ELB139, in contrast to diazepam (Fernandes *et al*., 1999), retained its significant effect on anxiety-related behaviour, even after chronic administration, indicating that it does not induce tolerance to its anxiolytic activity at least with the administration schedule used in the present experiment.

In summary, in the present study ELB139 elicited strong effects on anxiety-related behaviour mediated predominantly by its benzodiazepine-like activity. The extent of anxiolytic activity was comparable to that of diazepam. At efficacious doses the compound was devoid of major side-effects such as development of tolerance or sedation in rats. These characteristics make the compound a prime candidate for new anxiolytic drug. Indeed, the drug is currently undergoing phase II clinical testing as an anxiolytic.

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Footnotes

[#] The study was supported by the European Fund for Regional Development 2000-2006, sector technology support, and by the Federal State of Saxony (grant no. SAB 8093).

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Legends for Figures

Fig. 1. Chemical structure of ELB139.

Fig. 2. Effect of 1, 10 and 100 µM ELB139 on GABA-induced currents.

The GABA-induced current elicited by 3 µM GABA was set to 100% and the percentage of the current elicited by the substance during GABA application was calculated. Effects were tested for significance using the paired *t* test.

Fig. 3. Flumazenil antagonism of GABA-induced currents.

GABA or GABA and substance (10 µM) were supplied for 10 s followed by a washout of 30 s. Flumazenil (10µM) was administered 30 sec before the application of additional GABA and the test substance (ELB139 or diazepam). The currents were normalised to the current elicited by GABA (100%). Effects were tested for significance by the paired *t* test (*** $p < 0.001$).

Fig. 4. Results from the elevated-plus-maze after acute administration.

ELB139 at 10 and 30 mg/kg and diazepam at 6 mg/kg were administered once p.o. 1 h and 30 min, respectively, before the test. Rats were placed on the elevated-plus-maze for 10 min ($n = 12$). Data are shown as mean ± SEM. Significant to control: * = $p<0.05$ and *** = $p<0.001$; significance between 10 and 30 mg/kg ELB139, # = $p<0.05$.

Fig. 5. Results of the elevated-plus-maze after repeated administration.

Subacutely, ELB139 at 10 and 30 mg/kg and diazepam at 4 mg/kg (diaz) were administered p.o. twice daily for 4 days and 1 h or 30 min, respectively, before the test ($n = 5$). Chronically, ELB139 at 10 and 30 mg/kg p.o. was administered twice daily 5 days/week for 6 weeks ($n = 10$). Rats were placed on the elevated-plus-maze for 10 min. Data are shown as mean ± SEM. Significance: * = $p<0.05$.

Fig. 6. Reversal of the acute anxiolytic effect of ELB139 and diazepam by flumazenil.

Reversal of the acute anxiolytic effect of 4 mg/kg p.o. diazepam (D) and 30 mg/kg p.o. ELB139 (139) by flumazenil (F; 5 mg/kg i.p. 20 min before testing) in the elevated-plus-maze. Data are shown as mean ± SEM. Statistical significance was determined by one-way ANOVA and Tukey testing.

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Significantly different from control with $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***); # = significant difference between compound group and flumazenil group, $p < 0.05$.

Fig. 7. Results of the Vogel conflict test.

6 mg/kg diazepam and 10 and 30 mg/kg p.o. ELB139 were administered orally, respectively 30 min and 1 h before testing. Data are shown as mean ± SEM, * = significant, $p < 0.05$.

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Tables

Tab. 1: Results of the open field

compound	**dose p. o.** [mg/kg]	**Pre-treatment time** [h]	**n** [n]	mean ± SEM **activity** [sec]	**distance travelled** [m]	**rearings** [n]	**distance travelled in the centre** [%]	**time spent in the centre** [%]
Control		1	12	179.8 ± 29.4	53.4 ± 11.5	23.4 ± 5.8	5.2 ± 0.9	2.8 ± 0.9
Diazepam	6	1	12	239.3 ± 13.9	81.3 ± 9.5	31.3 ± 4.6	9.8 ± 1.5	5.1 ± 1.2
ELB139	10	1	12	178.8 ± 19.0	53.8 ± 7.2	24.1 ± 4.5	7.8 ± 1.3	2.7 ± 0.7
	30	1	12	159.9 ± 19.0	54.1 ± 9.9	17.1 ± 3.3	9.3 ± 1.8	3.9 ± 1.4

30 min after diazepam and 1h after ELB139 administration, rats were placed in the open field for 10 min. Data are shown as mean ± SEM.

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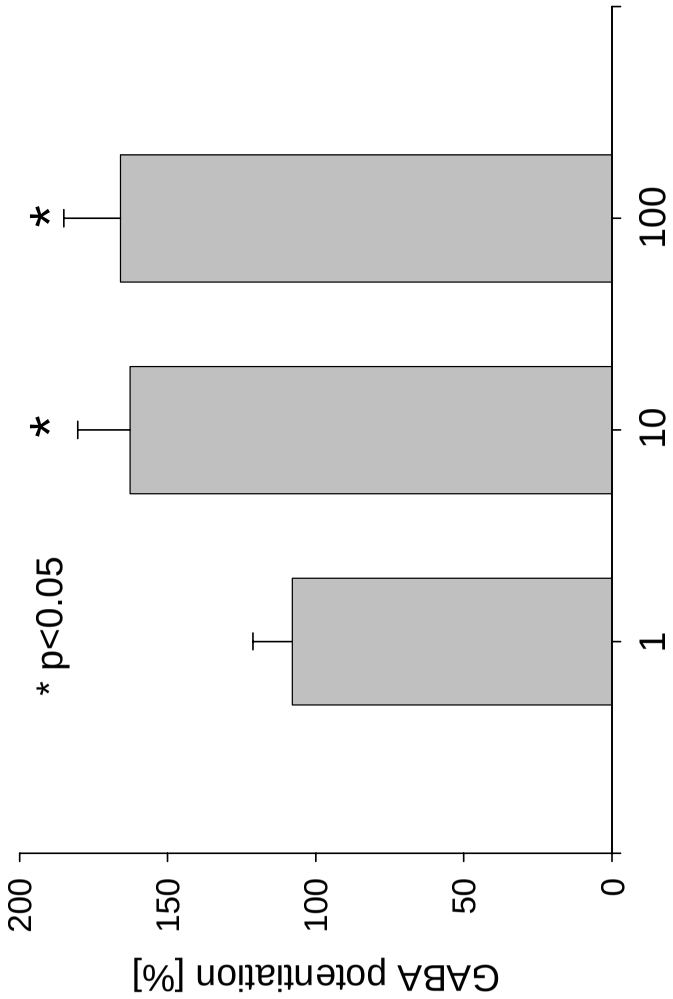

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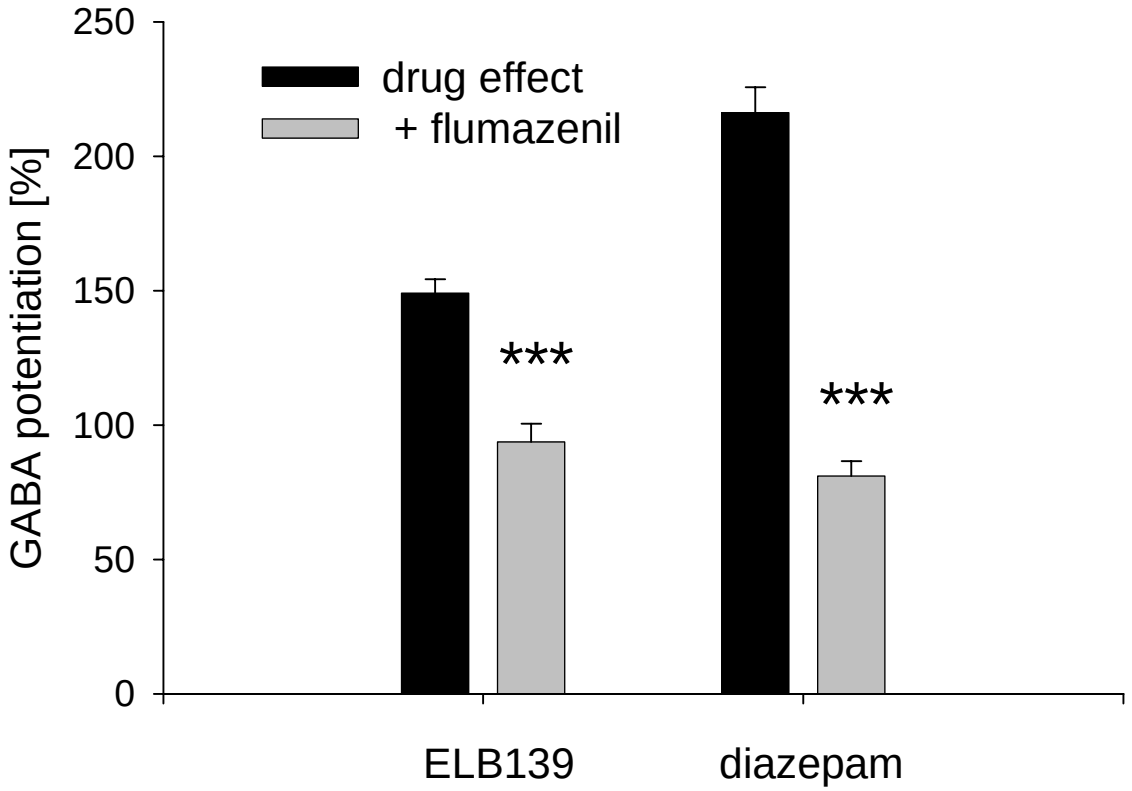

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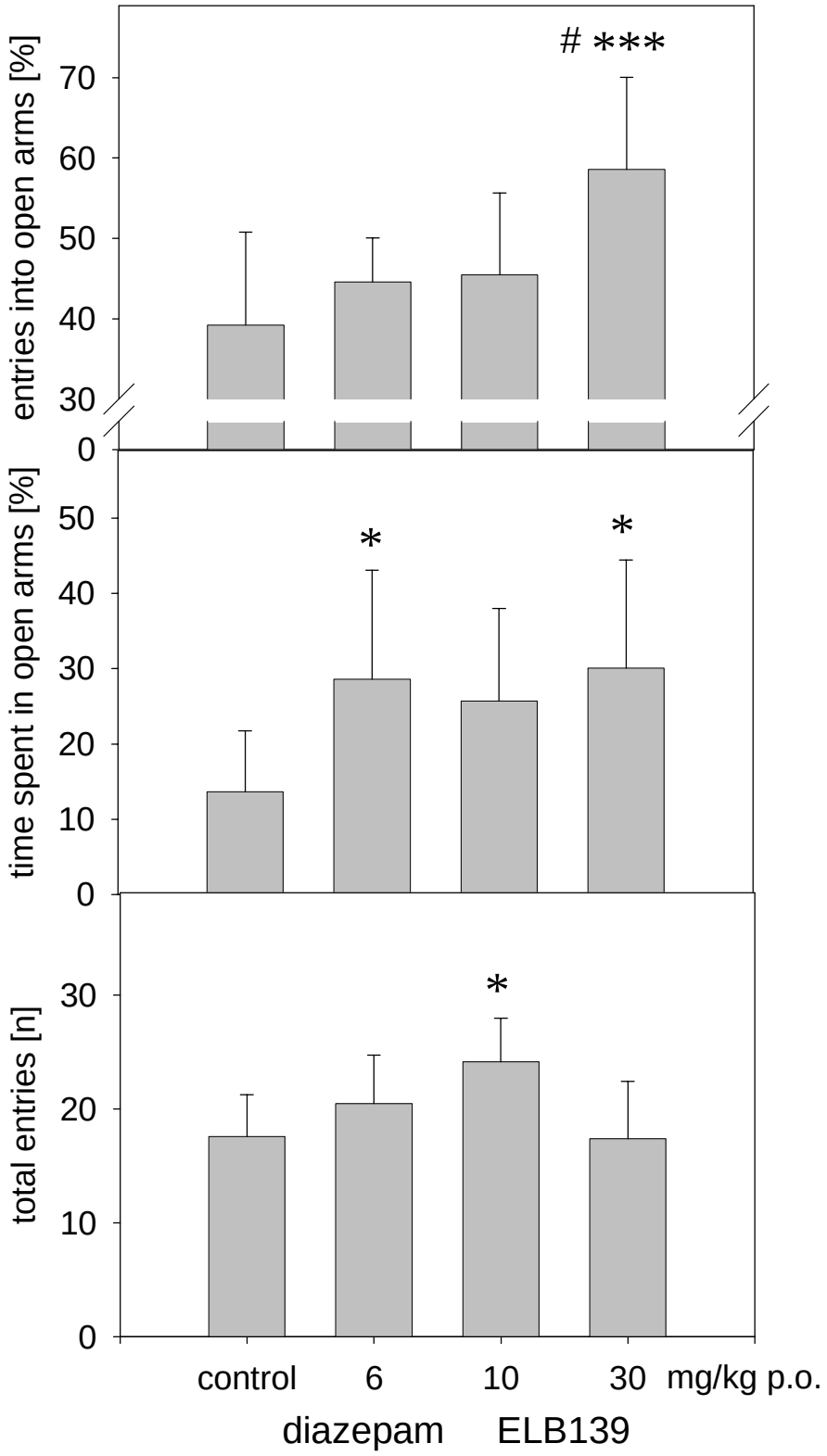

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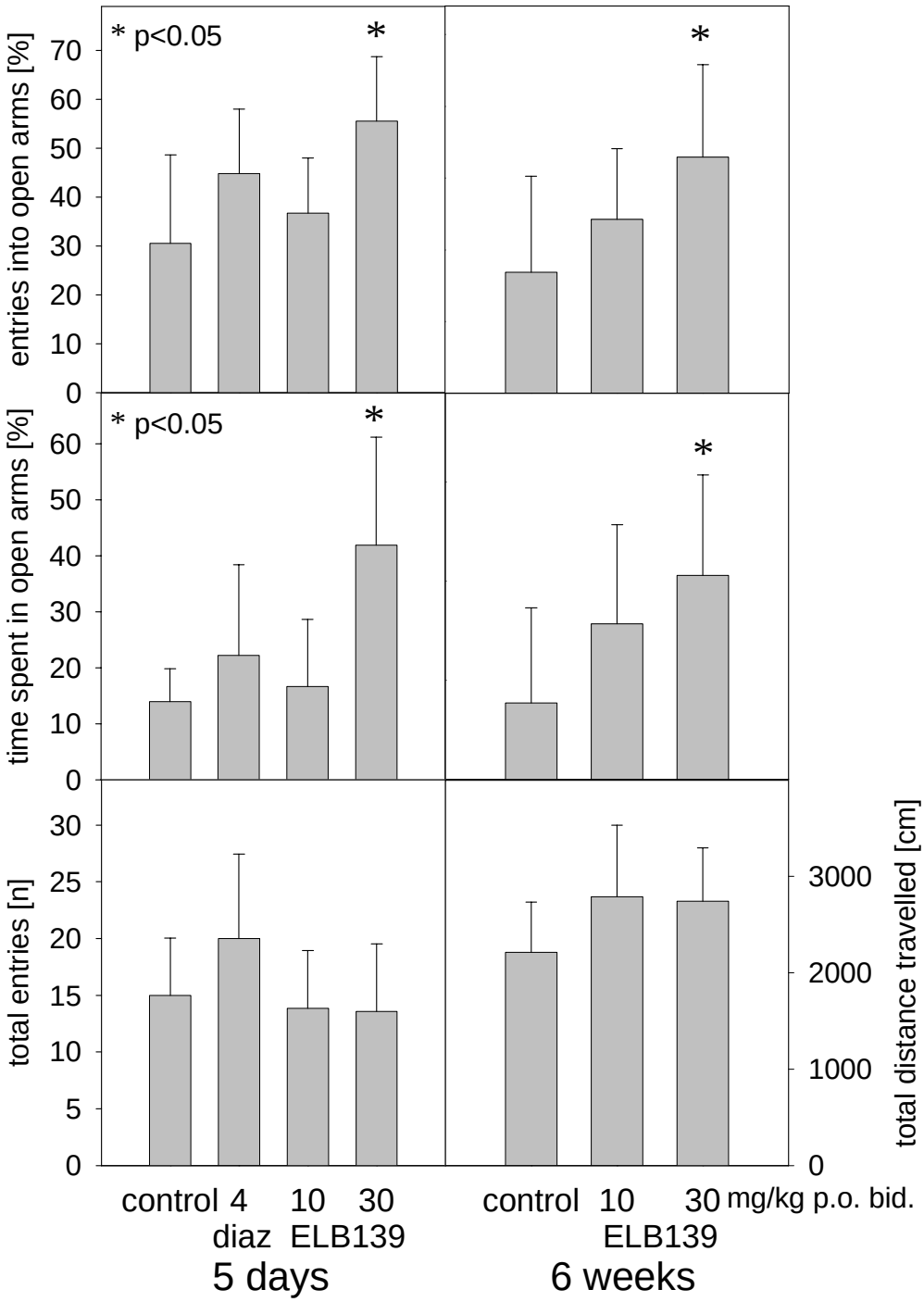

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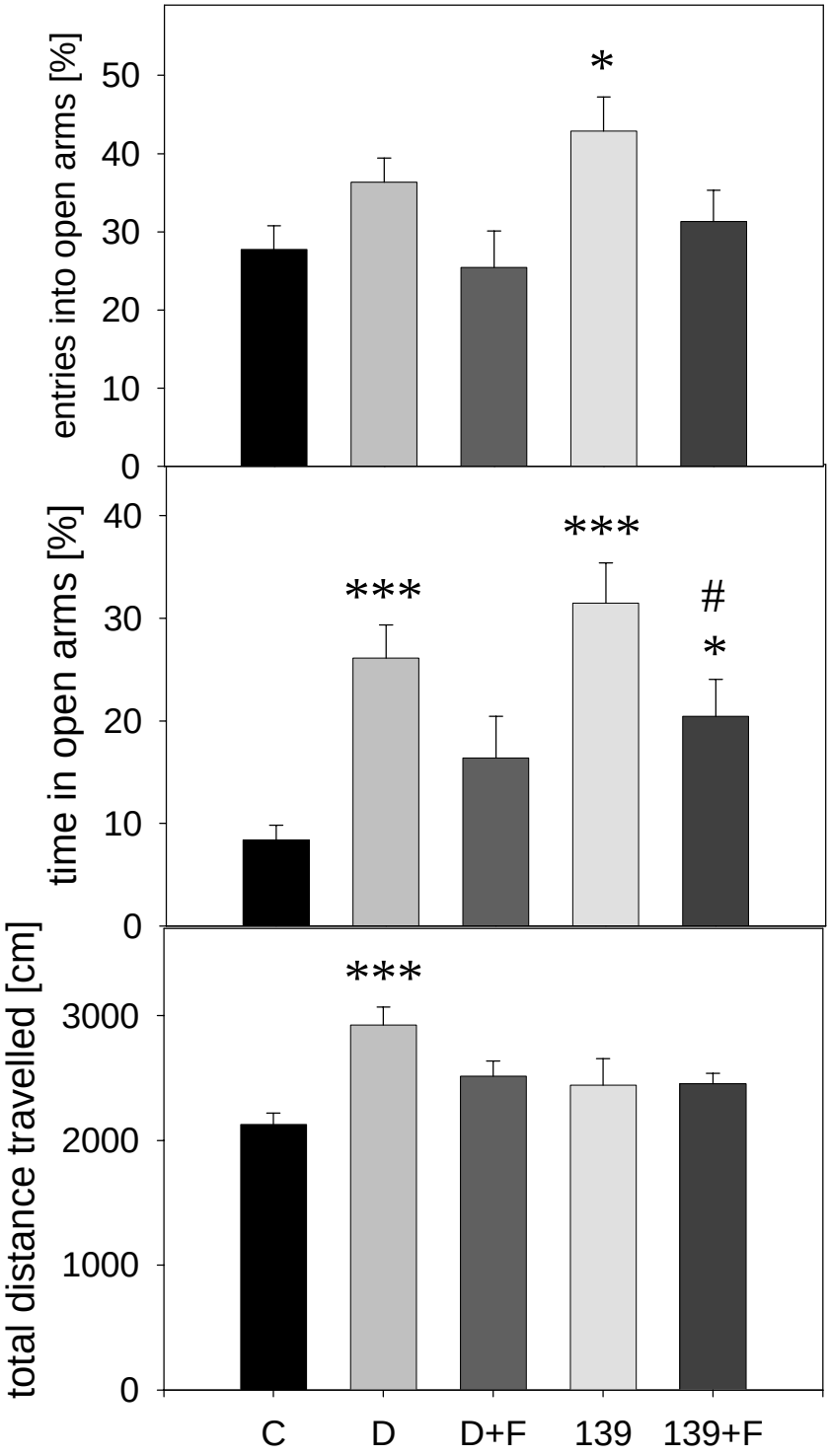

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