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Different effects of diazepam in Fischer rats and two stocks of Wistar rats in tests of anxiety

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Abstract

The behaviour of animals in tests of anxiety varies between strains, even in identical tests and surroundings. To evaluate the results obtained, a more detailed knowledge of the behaviour of different rat strains is indispensable. Identically raised Fischer 344 rats and two stocks of Wistar rats were examined in two animal tests of anxiety: the X-maze and a modified open-field test following diazepam treatment (0.5–4.0 mg/kg). Harlan–Wistar rats were the least 'anxious' when the behaviour of vehicle treated controls was compared. The largest effect of the anxiolytic diazepam, however, was observed in Harlan–Fischer rats. To determine possible reasons for strain and stock differences, plasma concentrations of diazepam and metabolites and concentrations of serotonin (5-HT) in the CNS were measured. Plasma concentrations of diazepam and metabolites differed between the strains with the Harlan–Fischer rats showing higher diazepam concentrations. 5-HT levels in discrete brain regions varied with Harlan–Fischer rats having higher 5-HT concentrations. Strain differences influence the anxiety-associated behaviour of untreated animals and the effect of anxiolytics.

Keywords: Anxiety; Diazepam; Metabolites; Open field; Serotonin; Strain differences; X-maze

1. Introduction

Studies in behavioural pharmacology have shown different and, sometimes, contradictory results using the same behavioural procedure or animal test, e.g., in cognitive behaviour (Van der Staay and Blokland, 1999), anxiety-related behaviour (Griebel, 1995; Hogg, 1996) and exploratory behaviour (Nakagawara et al., 1997). In a previous study, the anxiety-related behaviour of different rat strains raised under identical environmental conditions showed distinct baselines of anxiety-related behaviour and differences in the aversion-induced serotonin (5-HT) release in the hippocampus on exposure to the elevated plus maze (X-maze) (Rex et al., 1999). These strain differences in basal anxiety-related behaviour could influence the efficacy of 'anxiolytic' or 'anxiogenic' drugs reported in the literature.

To minimize environmental effects, in this study, male Fischer 344 and Wistar rats were obtained from the same breeder and a second stock of Wistar rats was obtained from a different vendor directly after weaning and then all rats were raised identically. They were tested in the elevated plus maze test, a well-established model of anxiety, based on elevation, novelty and open space as aversive stimuli and in a modified open-field test, based upon the suppression of feeding by exposure to a novel and aversive environment (Bodnoff et al., 1989; Britton and Britton, 1981).

Benzodiazepines are the most widely used anxiolytics in general practice for many years and are relatively safe drugs for a short-term treatment of anxiety despite their drug dependence potential and side effects (Ballinger, 1990). Since benzodiazepines are used as a "gold standard" for an anxiolytic drug (e.g., Hogg, 1996), we looked for probable strain-inflicted differences in anxiety-related behaviour after an acute treatment with diazepam. Diazepam is the standard anxiolytic employed in behavioural pharmacology, even if the screened drug is not acting via benzodiazepine receptors.

To assess possible differences in the pharmacokinetics of diazepam between the rat strains and stocks, we determined the plasma concentrations of diazepam and the three metabolites at the time of testing.

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The neurotransmitter 5-HT plays a crucial role in anxiety-related behaviour in animals and humans (e.g., Chopin and Briley, 1987). Drugs acting at various serotonergic receptors and decreasing or increasing the activity of the central serotonergic system have 'anxiolytic' or 'anxiogenic' effects in animals and man (Griebel, 1995). Exposure of animals to an animal test of anxiety as the X-maze or the social interaction test causes an increase in hippocampal 5-HT release monitored by microdialysis (Voigt et al., 1999; Marsden et al., 1995). This rise in extracellular 5-HT could be attenuated by administration of anxiolytic drugs (e.g., diazepam) before the exposure to the test (Rex et al., 1993), indicating a central interaction between the serotonergic transmission system and GABA-ergic mechanisms.

The aim of our study was the determination of variations in the anxiolytic effects of diazepam in different rat strains and, subsequently, the assessment of possible reasons for strain-dependent differences in the diazepam effects.

2. Material and methods

2.1. Animals

Male Fischer 344 rats (F344/NHsd, Harlan, Germany), Wistar rats (HsdCpb: WU, Harlan) and Wistar rats from the Federal Institute for Health Protection of Consumers and Veterinary Medicine (Han: WIST (SYN WI), FIHV, Germany) were purchased after weaning (Day 21) and then raised under identical environmental conditions in our animal unit. The rats were used at a weight of 220 ± 30 g (Age 8–11 weeks). They were group-housed, five per cage ($45 \times 60 \times 25$ cm), at room temperature (22 °C) and with a 12-h light–dark cycle (light on at 06:00 h) under moderate lighting levels (≈ 230 lx) (Hogg, 1996). Standard pellet food (Altromin 1326) and water were freely available. The tests were performed in a sound proofed chamber ($180 \times 180 \times 230$ cm) between 08:00 and 11:00 h. Rats from the strains were assigned randomly to the procedure. Group size was 9–13 animals.

One hour before the test was started the animals were transferred in their home cages from the animal unit to the observation chamber. After this habituation time, the rats received either diazepam (0.5–4.0 mg/kg) (Arzneimittelwerk Dresden, Germany) or vehicle (saline containing ethanol (186 mg/10 ml) and Macrogol 400) intraperitoneally. The injection volume was 1 ml/kg body weight. Thirty minutes later, the animals were subjected to the test procedure.

All performed procedures and experiments were approved by the animal protection board of the state of Berlin "Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin, Fachgruppe Veterinärwesen, Lebensmittelwesen und Gentechnik."

2.2. X-maze

The behavioural experiments were performed using an X-maze illuminated with 210 lx on the surface of the open arms, 190 lx in the centre and 60 lx in the closed arms. The X-maze was 64 cm high with four arms (44×15 cm; the wide arms beneficial for the use of larger rats), with a wall on two opposite arms (height: 15 cm). The animals were placed on the centre of the X-maze 30 min after receiving vehicle or diazepam. The experiments were performed for 5 min (Pellow et al., 1985; Rodgers and Cole, 1994). The animals were observed from the outside by a video camera suspended above the X-maze and the behaviour recorded and analyzed with a computerised automated tracking system (VideoTrack, CPL-Systems, UK). The behavioural parameters measured were: the number of entries into the closed arms (CE) and the open arms, the time spent in the open arms, the number of head dips over the edge of the open arms, the number of stretched attended postures from the protecting closed arms into the more aversive open arms without leaving the former (SAP) and the number of rearings (R). The percentage of entries into open arms of all entries was calculated. It is known that diazepam administered in a single dose may tend to depress motor activity. To exclude false positive anxiolytic effects the number of entries into all arms (TE) and the total distance travelled in metres were determined as measures of locomotor activity. If the locomotor activity decreased following treatment with higher doses diazepam, the series was terminated.

2.3. Modified open field

Twenty hours before testing, the pellets were removed from the animal's home cage; the water was still available. The rats were placed in a corner of the brightly illuminated (350 lx) white open field ($100 \times 100 \times 40$ cm) facing the centre of the open field. Familiar food pellets were placed in the centre of the open field. Each rat was observed for 5 min, and the onset of feeding (latency to start feeding) was recorded and the incidence of food intake (% of rats feeding in a group with the same treatment) was registered. If the feeding rate decreased following treatment with higher doses of diazepam after an observed increase in lower doses, the series was terminated.

The animals were observed using the same real-time video system as described above. Measurement of locomotor activity was carried out simultaneously by counting interruptions of 10 crosswise arranged infrared light beams in the open field.

2.4. Plasma concentrations of diazepam and the three metabolites

All animals received a single dose of diazepam (0.5, 1.0 or 2.0 mg/kg ip). After 30 min, the animals were anaes-

thetized with isoflurane and intracardial blood samples were taken. The blood was centrifuged at 4000 rpm at 0 °C for 10 min and the plasma transferred to a plastic vial and stored at − 80 °C.

Before extraction of diazepam and its metabolites, nordiazepam, temazepam and oxazepam, the plasma was mixed with 50 ml of a 100-ng/ml chlordiazepoxide solution in methanol/water (50/50) as internal standard and 50 ml of a 0.125-M sodium tetraborate solution (pH = 9.5). Diazepam and the metabolites were extracted with dichloromethane (1 ml).

The plasma concentrations of diazepam and the metabolites were measured by HPLC with UV detection (extinction of 232 nm) using a 20-cm Inertsil ODS-2-Column (5 μm, ∅ 4 mm, with a 1-cm precolumn, ∅ 2 mm, VDS Optilab, Germany) at a flow rate of 0.25 ml/min. The mobile phase consisted of sodium phosphate 20 mM (pH = 4.6), acetonitrile and isopropanol (volume distribution: 680/170/150 ml) at a temperature of 40 °C. Retention times were: oxazepam, 11.7 min; temazepam, 14.5 min; nordiazepam, 19.7 min; and diazepam, 25.6 min. For the analysis of the chromatograms, a data system CSW (DATA Apex, ČSR) was used. The concentrations are expressed in nanograms per milliliter of blood plasma.

2.5. Determination of 5-HT tissue levels

The rats received either diazepam 2.0 mg/kg or vehicle, and 30 min later, the animals were sedated with isoflurane (Forene, Abbott, Germany) for a minute, decapitated and the whole brains cautiously removed. The entire brain, stored in a plastic vial, was frozen in fluid nitrogen immediately. Later the brain was cut into sagittal slices. Slices (1 mm thick) of the prefrontal cortex (bregma 3.2 mm), the hippocampus (bregma − 5.8 mm) and the medial/dorsal raphe nucleus (bregma − 7.8 mm; Paxinos and Watson, 1997) were stamped in both hemispheres (∅ 1 mm) with a trocar (Hauptner, Germany).

The two samples of a region were immersed in ice chilled with 0.1 M perchloric acid (600 μl) and homogenised. The protein concentration in the homogenised solution was determined in 200 μl by the Lowry assay for protein concentration (Lowry et al., 1951). The other 400 μl were centrifuged for 10 min at 14,000 rpm at 5 °C. In the supernatant, the concentration of 5-HT was determined by HPLC with electrochemical detection using a 12.5 cm GROM-Symbasic C18 column (GROM, Germany) (5 μm, ∅ 4 mm) at a flow rate of 1 ml/min. The mobile phase consisted of 0.1 M NaH_2PO_4, 1 mM

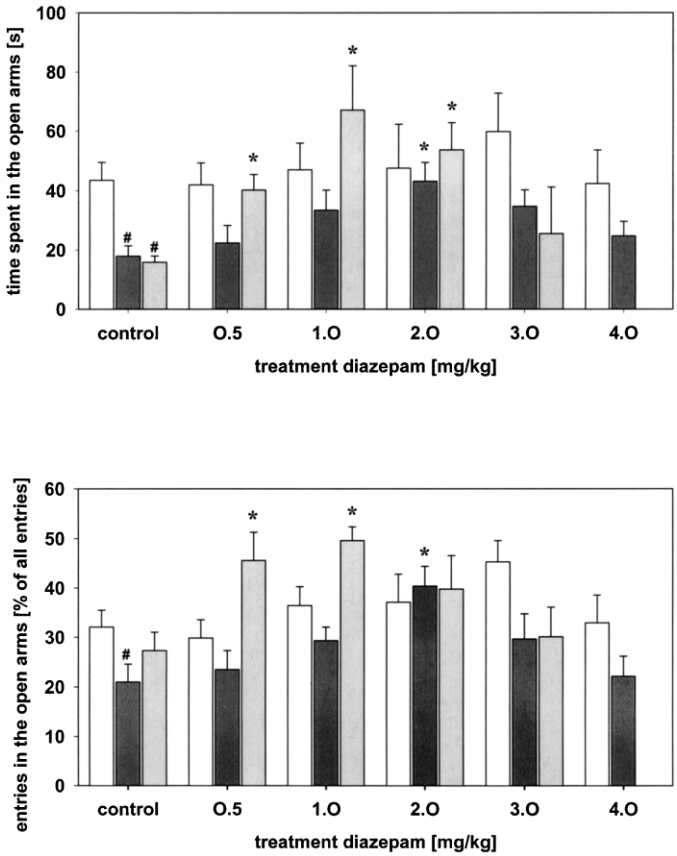


Fig. 1. Time spent and entries in the open arms of the X-maze in the Harlan–Wistar rats (□), the FIHV–Wistar rats (■) and the Harlan–Fischer rats (▩) following an injection of diazepam (ip). A two-way ANOVA followed by post hoc Students–Newmann–Keuls test ($^{\#}P < .05$ controls pairwise, * $P < .05$ treatment vs. control) was used. Data were presented as means ± S.E.M.

EDTA-disodium salt, 0.73 mM octane sulphane acid sodium salt (pH = 5.35) and 3% isopropanol. For the analysis of the chromatograms, a data system CSW (DATA Apex) was used. The concentrations of 5-HT were expressed in millimoles per milligram protein.

2.6. Statistics

Statistical analysis (except feeding data) was performed with a two-way ANOVA followed by post hoc analysis using the Student–Newman–Keuls test. Data are presented as means ± S.E.M. The feeding data from the modified open-field test were analysed using a chi-squared test. These data are shown as percent. Differences of the means, $P < .05$, were considered as statistically significant.

3. Results

3.1. X-maze

On exposure to the X-maze, the anxiety-related behaviour of the three rat strains differed markedly, and there was a statistically significant interaction between the strains and the diazepam treatment. The Harlan–Wistar rats spent 41.7 ± 5.9 s [$F(10,204) = 2.562$, $P = .06$] in the open arms and 31.0 ± 3.4% [$F(10,204) = 5.801$, $P < .001$] of all entries were directed into the open arms (Fig. 1). The rats bend 9.8 ± 1.4 [$F(10,204) = 4.074$, $P < .001$] times downward over the edge of the open arms (head dips) (Fig. 2). The Harlan–Fischer rats spent less time in the open arms ($P = .03$; 14.4 ± 2.2 s) but had no lower percentage of entries (27.3 ± 3.7%) than the Harlan–Wistar rats (Fig. 1). During the exploration in the open arms, the Harlan–Fischer rats showed fewer head dips than the Harlan–Wistar rats ($P = .002$; 5.2 ± 0.6 head dips) (Fig. 2).

The FIHV–Wistar rats spent less time in the open arms ($P = .03$; 17.9 ± 3.5 s) than the Harlan–Wistar rats and tended to enter the open arms less often (20.9 ± 3.6%) (Fig. 1). The FIHV–Wistar rats showed fewer head dips than the Harlan–Wistar rats ($P = .002$; 4.6 ± 0.7 times) from the open arms (Fig. 2). The number of SAP [$F(10,204) = 3.210$, $P < .001$] in the three rat strains differed between the Harlan–Fischer rats (1.5 ± 0.3) and the Harlan–Wistar rats (2.7 ± 0.5; $P = .007$) (Fig. 2).

There was no difference in the number of entries into the closed arms (CE) or in the number of rearings (R) between the vehicle treated Harlan–Wistar rats (7.77 ± 0.86 CE; 19.66 ± 1.43 R), the FIHV–Wistar rats (5.24 ± 1.01 CE; 15.25 ± 1.32 R) or the Harlan–Fischer rats (5.76 ± 0.89 CE; 15.56 ± 1.55 R) (Table 1).

The rat strains differed neither in the number of total entries (TE) on the X-maze nor in the distance travelled during the test: Harlan–Wistar: (11.44 ± 1.23 TE; 8.8 ± 0.7 m), FIHV–Wistar: (7.29 ± 1.1 TE; 7.6 ± 0.9 m), Harlan–Fischer: (7.21 ± 1.1 TE; 7.5 ± 0.8 m) (Table 1). The effect of diazepam on the anxiety related behavioural parameters on exposure to the X-maze depended on the rat strain used.

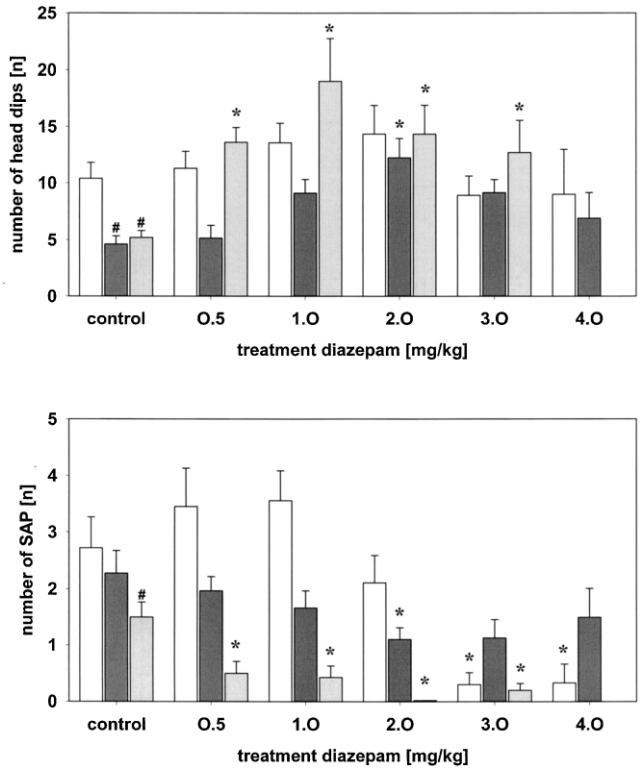


Fig. 2. Number of head dips and number of SAP on the X-maze in the Harlan–Wistar rats (□), the FIHV–Wistar rats (■) and the Harlan–Fischer rats (▩) following an injection of diazepam (ip). A two-way ANOVA followed by post hoc Students–Newmann–Keuls test ($^{\#}P < .05$ controls pairwise, $*P < .05$ treatment vs. control) was used. Data were presented as means ± S.E.M.

Following the treatment with diazepam, we could measure 'anxiolytic' effects on the X-maze in the Harlan–Fischer rats and in the FIHV–Wistar rats but no effects in the Harlan–Wistar rats, compared to the vehicle-treated rats. The percentage of entries into the open arms was doubled in the Harlan–Fischer rats, reaching a maximum at a diazepam dose of 1.0 mg/kg ($P < .001$; 49.6 ± 4.1%), and in the FIHV–Wistar rats, peaking at diazepam 2.0 mg/kg ($P = .006$; 40.4 ± 5.8%), compared to the respective controls (Fig. 1). The time spent in the open arms increased approximately threefold in the Harlan–Fischer rats ($P < .001$; 67.4 ± 15.0 s) and the FIHV–Wistar rats ($P = .048$; 43.1 ± 9.2 s), with maximal effects at the same doses, respectively (Fig. 1). A similar change could be seen in the indicators for a risk taking as the number of head dips in the Harlan–Fischer rats ($P < .001$; 19.0 ± 3.8) and the FIHV–Wistar rats ($P = .002$; 12.2 ± 1.7) (Fig. 2). The frequency of SAP, another parameter of risk assessment, was decreased in both the Harlan–Fischer rats ($P = .039$; 0.5 ± 0.21) and the FIHV–Wistar rats ($P = .045$; 1.11 ± 0.25) starting at 0.5 and 2.0 mg/kg, respectively (Fig. 2).

The number of rearings [$F(10,204) = 3.262$, $P < .001$], an indicator of vertical exploration, was decreased in the Harlan–Fischer rats (7.7 ± 1.4, $P < .001$) starting at 2.0 mg/kg and Harlan–Wistar rats (6.7 ± 2.2, $P < .001$) starting at 3.0 mg/kg, but not in the FIHV–Wistar rats (Table 1).

Diazepam did not influence the number of entries into the closed arms in the Harlan–Fischer rats or the FIHV–Wistar rats (Table 1).

In contrast, in the Harlan–Wistar rats diazepam did not change the anxiety-related behaviour significantly. Only at the dose of 3.0 mg/kg diazepam the percentage of entries into the open arms (Fig. 1) and following 2.0 mg/kg diazepam the number of head dips from the open arms tended to increase (Fig. 2). The number of SAP decreased following administration of 3.0 and 4.0 mg/kg to 0.30 ± 0.21 SAP ($P < .001$) and 0.33 ± 0.30 SAP ($P < .001$), respectively (Fig. 2).

Diazepam did not influence the number of entries into the closed arms in the Harlan–Wistar rats, but the number of rearings declined following administration of 3.0 and 4.0 mg/kg to 6.67 ± 2.21 R ($P < .001$) and 5.67 ± 2.40 R ($P < .001$), respectively (Table 1).

Locomotor activity on the X-maze was affected [$F(10,204) = 2.846$, $P = .003$]. In Harlan–Wistar rats and the FIHV–Wistar rats, neither the number of total entries nor the distance travelled throughout the experiment was changed profoundly by diazepam. Only in the Harlan–Fischer rats diazepam had stimulating effects following administration of 1.0 mg/kg (11.9 ± 1.4 m; $P = .028$), as well as sedative effects following administration of 3.0 mg/kg (3.1 ± 0.7 m; $P = .007$) seen in the total distance travelled, with the same tendency, but not significantly, in the number of total entries (Table 1).

3.2. Modified open field

In the modified open-field test, the strains showed differences in basal behaviour and, additionally, the effect of diazepam treatment depended on which strain is treated, indicating an interaction between diazepam treatment and rat strains.

The number of vehicle-treated rats feeding during the 5-min test session showed a substantial interstrain variability (Fig. 3). Most of the Harlan–Wistar rats (85%) were feeding in the open field. In the Harlan–Fischer rats and the FIHV–Wistar rats, the percentage of rats feeding in the aversive open field was substantially lower with only 30% ($P < .05$) and 35% ($P < .05$) of animals in a group feeding, respectively (Fig. 3). The latency to start eating was shorter in the Harlan–Wistar rats [123 ± 24 s; $F(10,204) = 6.730$, $P < .001$] than in the FIHV–Wistar rats (242 ± 19 s, $P < .001$) and in the Harlan–Fischer rats (249 ± 20 s, $P < .001$) (Fig. 3).

The rat strains differed also in the locomotor activity in the open field [$F(10,204) = 2.648$, $P = .005$]. The Harlan–Fischer rats crossed only 53.9 ± 11.2 light beams ($P < .001$) in the 5-min observation period and were therefore less active than the Harlan–Wistar rats (114.1 ± 9.2 light beam crossings). The activity of the FIHV–Wistar rats (94.3 ± 11.9 light beam crossings) differed not from the Harlan–Wistar rats (Table 1).

Diazepam induced in the Harlan–Fischer rats ($P = .001$) and the FIHV–Wistar ($P = .002$) a dose-dependent increase in the percentage of rats feeding followed by a decrease, resulting in an inverted U-shape dose–response curve, peaking at 1.0 mg/kg with 90% of the rats in a group feeding (Fig. 3). In the Harlan–Wistar rats diazepam did not

Table 1
Effects of diazepam on motor activity of the rats on exposure to the X-maze and in the open field, showing on the number of rearings, number of entries into the closed arms, number of total entries into all arms, total distance travelled on the X-maze and the number of photo beam crossings in the open field

			Diazepam (mg/kg)				
Parameter	Strain	Control	0.5	1.0	2.0	3.0	4.0
Rearing [*n*]	Harlan–Wistar	19.7 ± 1.4	17.3 ± 1.6	16.7 ± 1.7	15.1 ± 2.5	6.7 ± 2.2*	5.7 ± 2.4*
	FIHV–Wistar	15.6 ± 1.6	16.9 ± 1.2	17.2 ± 0.9	12.7 ± 1.8	11.7 ± 3.2	9.2 ± 4.6
	Harlan–Fischer	15.3 ± 1.3	14.5 ± 2.0	12.1 ± 1.9	7.7 ± 1.4*	2.0 ± 1.6*	–
Closed arm entries [*n*]	Harlan–Wistar	7.8 ± 0.9	8.3 ± 0.9	7.5 ± 0.9	8.3 ± 1.4	5.7 ± 0.9	6.8 ± 1.5
	FIHV–Wistar	5.8 ± 0.9	5.9 ± 0.9	7.6 ± 1.0	6.6 ± 0.9	7.5 ± 1.5	5.4 ± 1.0
	Harlan–Fischer	5.2 ± 1.0	7.0 ± 1.1	6.7 ± 1.5	6.3 ± 1.2	3.9 ± 1.3	–
Total entries [*n*]	Harlan–Wistar	11.4 ± 1.2	11.8 ± 1.3	11.9 ± 1.2	13.2 ± 2.0	10.4 ± 1.3	10.2 ± 2.1
	FIHV–Wistar	7.3 ± 1.1	7.8 ± 1.0	10.8 ± 0.9	11.1 ± 1.3	10.7 ± 1.3	7.0 ± 1.5
	Harlan–Fischer	7.2 ± 1.9	12.8 ± 2.0	13.3 ± 2.8	10.5 ± 2.2	5.6 ± 2.4	–
Total distance [m]	Harlan–Wistar	8.9 ± 0.7	9.0 ± 0.9	10.1 ± 0.9	10.0 ± 0.8	7.4 ± 1.3	7.0 ± 2.4
	FIHV–Wistar	7.6 ± 0.9	9.2 ± 0.8	9.9 ± 0.7	10.0 ± 0.9	10.0 ± 1.2	7.1 ± 1.9
	Harlan–Fischer	7.4 ± 0.8	10.1 ± 0.6	11.9 ± 1.4	8.6 ± 0.9	3.1 ± 0.7	–
Photobeam crossings [*n*]	Harlan–Wistar	114.1 ± 9.2	108.2 ± 13.4	116.8 ± 12.8	68.3 ± 12.4	58.8 ± 11.5*	51.6 ± 15.2*
	FIHV–Wistar	94.3 ± 11.9	98.6 ± 13.6	127.1 ± 18.5	66.9 ± 15.2	29.9 ± 7.1*	15.3 ± 8.5*
	Harlan–Fischer	53.9 ± 11.2**	145.4 ± 22.1*	100.1 ± 19.9	57.7 ± 16.7	15.6 ± 5.3*	–

A two-way ANOVA followed by post hoc Students–Newmann–Keuls test was used. Data were presented as means ± S.E.M.

* $P < .05$, treatment vs. control.

** $P < .05$, controls pairwise.

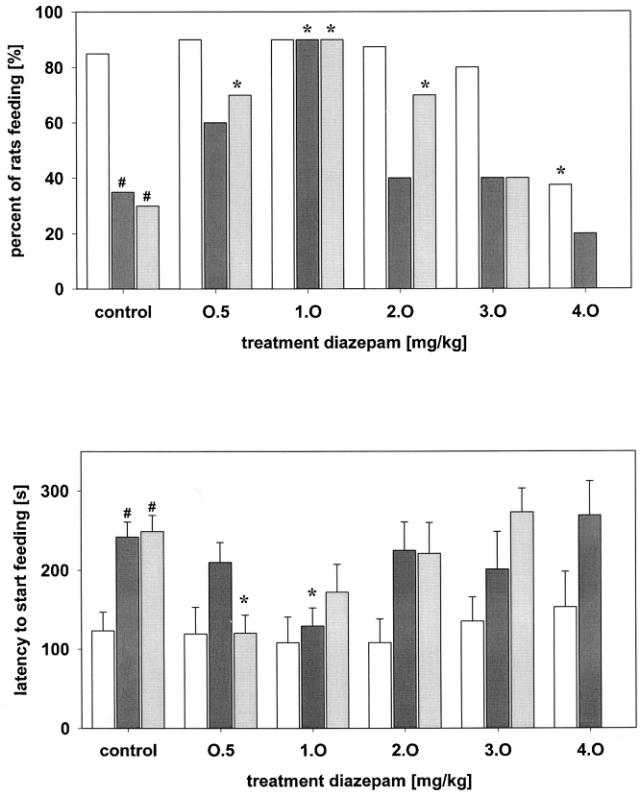


Fig. 3. Percentage of rats feeding and latency to start feeding in the modified open-field test in the Harlan–Wistar rats (□), the FIHV–Wistar rats (■) and the Harlan–Fischer rats (▩) following an injection of diazepam (ip). A two-way ANOVA followed by post hoc Students–Newmann–Keuls test ($^{\#}P<.05$ controls pairwise, $*P<.05$ treatment vs. control, latency to start feeding; $^{\#}P<.05$ controls pairwise, $*P<.05$ treatment vs. control, chi-squared test, rats in a group feeding) was used. Data were presented as percent (rats in a group feeding) and means ± S.E.M. (latency to start feeding).

increase the number of rats feeding, because 80% of the vehicle-treated rats were already feeding, but in the highest dose of diazepam (4.0 mg/kg), the number of rats feeding was decreased (37.5%; $P=.008$).

The latency to start feeding was reduced in the Harlan–Fischer rats (120.0 ± 22.8 s; $P=.015$) and the FIHV–Wistar (129.0 ± 22.9 s; $P=.009$) following the administration of 0.5 and 1.0 mg/kg diazepam, respectively (Fig. 3). In the Harlan–Wistar rats, the latency to start feeding was not changed following the pretreatment with diazepam.

The locomotor activity was decreased in Harlan–Wistar rats and the FIHV–Wistar rats following treatment with diazepam, starting at 2.0 mg/kg (68.3 ± 12.4 light beam crossings; $P=.025$) and 3.0 mg/kg (29.9 ± 7.1 light beam crossings; $P=.002$), respectively. In the Harlan–Fischer rats, diazepam (0.5 mg/kg) induced a hyperlocomotion (145.4 ± 22.1 light beam crossings; $P<.001$) and at a higher dose of 3.0 mg/kg a sedative effect (15.6 ± 5.3 light beam crossings; $P=.014$).

3.3. Plasma concentrations of diazepam and the three metabolites

To mimic the diazepam concentrations during the behavioural test, the plasma concentrations of diazepam and the three metabolites were determined 30 min following the administration of diazepam (0.5, 1.0 or 2.0 mg/kg).

Following the treatment with diazepam 0.5 mg/kg, there were no differences in the plasma concentrations of diazepam in all three rat strains observed.

After treatment with diazepam 1.0 and 2.0 mg/kg, the concentrations of diazepam [$F(4,86)=7.132$, $P<.001$] and the three metabolites, nordiazepam [$F(4,86)=22.523$, $P<.001$], temazepam [$F(4,86)=39.280$, $P<.001$] and oxazepam [$F(4,86)=2.673$, $P=.038$], varied between the strains, indicating an interaction of the treatment and rat strains. The diazepam concentrations were significantly increased in the Harlan–Fischer rats ($P=.043$) in comparison to the Harlan–Wistar rats. Diazepam, starting at 0.5 mg/kg, caused an increase in the three metabolites [oxazepam ($P<.001$), temazepam ($P<.001$) and nordiazepam ($P<.024$)] in the Harlan–Fischer rats in comparison to the Harlan–Wistar rats. Between the two stocks of Wistar rats, we could not find significant differences (Fig. 4).

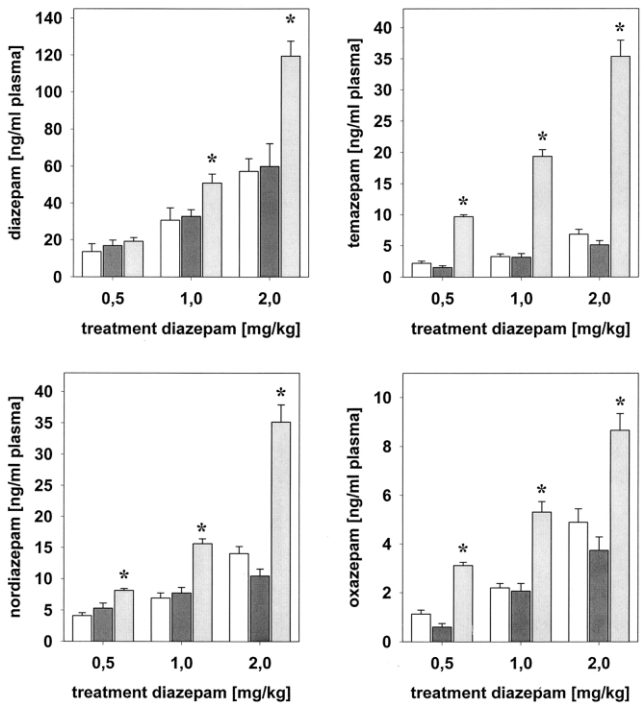
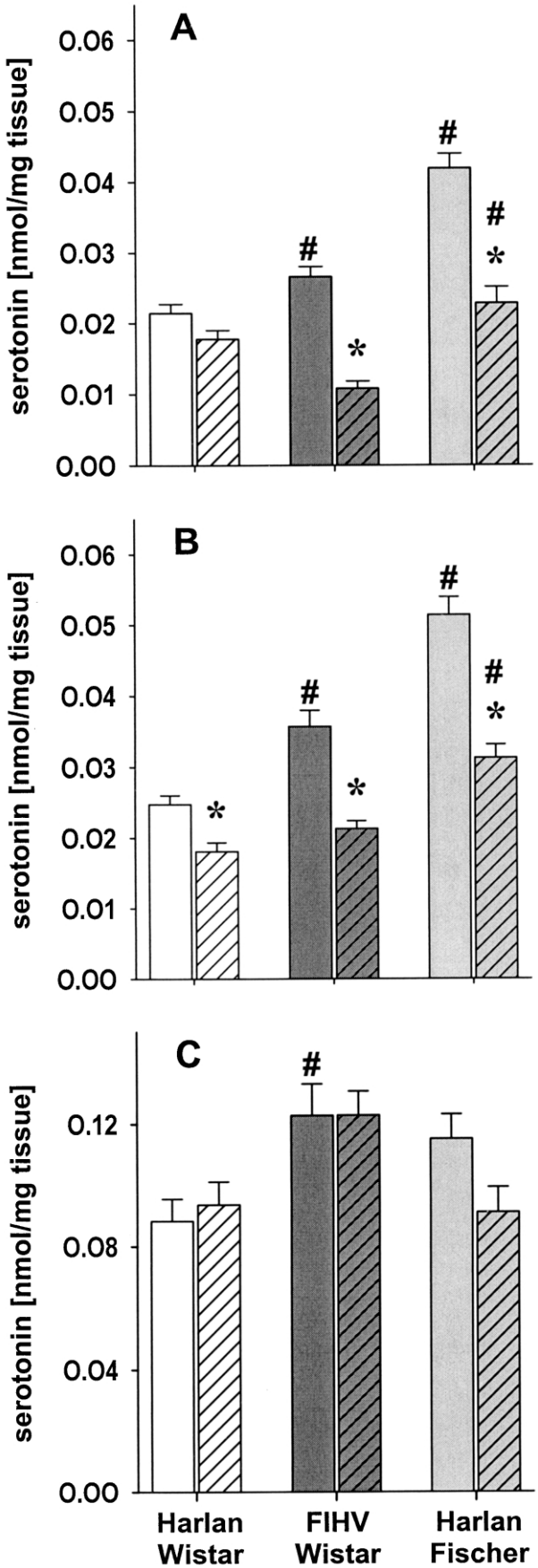


Fig. 4. Concentrations of diazepam and the three metabolites, nordiazepam, temazepam and oxazepam, in blood plasma of the Harlan–Wistar rats (□), the FIHV–Wistar rats (■) and the Harlan–Fischer rats (▩) following an injection of diazepam (ip), measured by HPLC. A two-way ANOVA followed by post hoc Students–Newmann–Keuls test ($^{\#}P<.05$ controls pairwise, $*P<.05$ treatment vs. control) was used. Data were presented as means ± S.E.M.



Additionally, following treatment with diazepam, the Harlan–Fischer rats metabolize diazepam (D) to nordiazepam (N) and temazepam (T) to the same extent (ratio D/N/T = 100:34.1 ± 4.1:39.4 ± 6.1; Table 1; $P = .02$), whereas in the Harlan–Wistar rats (ratio D/N/T = 100:25.7 ± 2.3:12.9 ± 1.7), temazepam could be detected only in lower ratios to diazepam and nordiazepam [$F(4,86) = 4.649$, $P = .012$]. The ratio of the metabolites in FIHV–Wistar rats (ratio D/N/T = 100:28.9 ± 2.7:9.1 ± 1.3) did not differ from the Harlan–Wistar rats.

3.4. Tissue concentrations of 5-HT in the CNS in untreated controls and diazepam treated rats

The tissue concentrations of 5-HT were determined in three discrete brain areas, related to anxiety and relevant for the serotonergic neurotransmission system. The analysis revealed strain-dependent effects of diazepam on the tissue concentrations of 5-HT in the in the prefrontal cortex [$F(2,59) = 7.253$, $P = .002$] and the hippocampus [$F(4,86) = 11.150$, $P < .001$].

The rats displayed a substantial interstrain variability in tissue concentrations of 5-HT in the CNS. In the hippocampus ($P < .001$) and the prefrontal cortex ($P < .001$), but not in the raphe region, the 5-HT concentrations were higher in the Harlan–Fischer rats than in the Harlan–Wistar rats.

5-HT concentrations in the FIHV–Wistar rats were higher in the hippocampus ($P = .048$) and the prefrontal cortex ($P = .015$), but not in the raphe region, compared to the Harlan–Wistar rats (Fig. 5).

Following treatment with diazepam, 5-HT levels in the hippocampus and in the prefrontal cortex were decreased markedly in the Harlan–Fischer rats ($P < .001$ and $P < .001$, respectively) and in the FIHV–Wistar rats ($P < .001$ and $P < .001$, respectively). In the Harlan–Wistar rats, diazepam decreased hippocampal 5-HT ($P = .004$) but not in the prefrontal cortex. Diazepam had no significant effect on 5-HT concentrations in the raphe region in all rat strains.

4. Discussion

Baseline behaviour of laboratory rats is markedly affected by various determinants, e.g., prior stress and handling, lighting conditions during the test and also in the animal unit, general conditions of housing, pretesting and time of testing (Dawson and Tricklebank, 1995; File and Day, 1972, Rodgers and Cole, 1994). In addition, raising or keeping animals under different environmental conditions may con-

Fig. 5. Concentrations of serotonin following an injection of diazepam (2.0 mg/kg, ///) or vehicle (□) (ip) in the prefrontal cortex (A), the hippocampus (B) and the raphe region (C), measured by HPLC. A two-way ANOVA followed by post hoc Students–Newmann–Keuls test ($^{\#}P < .05$ strain, $^{*}P < .05$ treatment vs. control) was used. Data were presented as means ± S.E.M.

tribute to differences in behaviour (Walsh and Cummins, 1976). 'Anxiety' and fear-related behaviour is also affected by gender and age of the animals used (File, 1992). To exclude environmental variables as a factor contributing to behavioural variability, all rats were raised in our animal unit after weaning.

However, it is known that different strains of rats even raised under identical conditions and studied in identical behavioural tests display discrete behaviour in anxiety tests. These differences found in untreated rats might explain the sometimes inconsistent results following the treatment with 'anxiolytic' or 'anxiogenic' substances as reported in literature (Hogg, 1996). Therefore, such strain differences in the behaviour of untreated animals could alter seemingly the efficacy of drugs.

In our study, especially the Harlan–Fischer rats and the Harlan–Wistar rats showed 'opposite' baseline levels of anxiety-related behaviour, with the Fischer rats appearing to be more 'anxious' than the Harlan–Wistar rats. These results are consistent with an earlier study using Fischer rats from Charles-River (Rex et al., 1996). Comparable differences in anxiety-related behaviour between Wistar rats and Fischer rats have been demonstrated earlier in models of anxiety as the social interaction test (Berton et al., 1997) and in tests of exploratory behaviour (Nakagawara et al., 1997). Interestingly, the two stocks of Wistar rats displayed different anxiety-related behaviour with the FIHV–Wistar rats showing a baseline behaviour similar to the Fischer rats, indicating robust differences between the outbred Wistar lines in anxiety-related behaviour. This may explain also the variability in the results of experiments using Wistar rats described in the literature.

Diazepam is a standard benzodiazepine anxiolytic and has been frequently employed in behavioural pharmacology as a reference compound.

The demonstration of anxiolytic effects of benzodiazepines is an essential aspect in the process of validating an animal model of anxiety.

In the present experiments, diazepam induced clear dose-dependent anxiolytic effects in the Harlan–Fischer rats and in the FIHV–Wistar rats in both tests for anxiety. Our results are in line with a number of previous reports describing the effects of diazepam on anxiety-related behaviour in the X-maze (for comparison, e.g., Dawson and Tricklebank, 1995; Hogg, 1996). Furthermore, diazepam is active in most animal tests of anxiolytic activity, including in a test measuring the consumption of novel unfamiliar food (Poschel, 1971), in a light–dark choice novelty situation (Merlo-Pich and Samanin, 1989), in the two compartment 'Black and White' box (Costall et al., 1988), in the social interaction test (Cadogan et al., 1994) and in the defensive burying paradigm (Rohmer et al., 1990).

In the Harlan–Wistar rats, diazepam had no anxiolytic effects; neither in the X-maze nor in the modified open-field test.

The most effective doses of diazepam were lower in the Fischer rats (1 mg/kg) compared to the Harlan–Wistar rats (3 mg/kg). Strain differences seem to have a large effect not only on the behaviour of untreated animals, but also on the effect of anxiolytic drugs (Liebsch et al., 1998). When we compared the maximal effects of diazepam on the classical parameters related to anxiety in the X-maze test, we observed a ceiling effect in all strains. The rats spent still a certain amount of time in the closed arms. This is plausible, since diazepam can reduce anxiety, but should not induce an aversion against the protecting closed arms of the X-maze. Likewise, it is impossible to increase the number of Harlan–Wistar rats feeding in the open field significantly. In general, it might be difficult to detect an anxiolytic effect in rats with an anxiolytic-like baseline behaviour as the Harlan–Wistar rats. Comparable results were observed in other rat strains on exposure to the X-maze (Ramos et al., 1997).

The differences in the most effective doses of diazepam can be explained by variations in pharmacokinetic parameters of the drug and by central mechanisms as variances in distribution (Liupina et al., 1999) and sensitivity (Hogg et al., 1996) of benzodiazepine binding sites between the strains or lines. Even interactions of central transmission systems, as the GABAergic system with other central neurotransmission systems, as the 5-HT system or with neuropeptides, which are also involved in the modulation of anxiety, could differ between the rat strains (Sudakov et al., 2001).

The determination of the plasma levels of diazepam and metabolites showed considerable differences between the two rat strains, with smaller differences within the Wistar rats. The higher concentrations of diazepam in the Harlan–Fischer rats at the testing time, compared to both Wistar stocks could be one reason for the anxiolytic effects of lower diazepam doses in the Harlan–Fischer rats.

Diazepam undergoes an extensive first-pass effect in the rat (Löscher and Frey, 1981) determined by the liver capacity. The high plasma concentrations of diazepam and all the three metabolites measured in the Harlan–Fischer rats suggest a higher bioavailability than in the Wistar rats. A possible explanation could be a reduced first-pass effect in the Harlan–Fischer rats in comparison to the Wistar rats, leading to a presystemic elimination of lesser extent and hence resulting in higher plasma concentrations. The marked differences in the plasma concentrations of diazepam following treatment with 1.0 and 2.0 mg/kg, but not after 0.5 mg/kg, could be an indicator for a saturation of the hepatic enzyme activity in the Fischer rats. The observed differences in the ratio of metabolite concentrations indicate an involvement of cytochrome *P*-450 isoenzymes as the major diazepam metabolizing enzyme. Mainly two forms of the cytochrome *P*-450, the CYP2C19 and the CYP3A (Jung et al., 1997), catalyze the N-dealkylation of diazepam to nordiazepam and the 3-hydroxylation of diazepam to temazepam, respectively. The different ratio of the concentrations

of nordiazepam and temazepam in the blood plasma of the Harlan–Fischer rats can be caused by a modified metabolism compared to the Wistar rats via the cytochrome *P*-450 (Shvarts et al., 1999). Genetic polymorphisms of the cytochrome *P*-450 are common and have the potential to affect a drug's action. The relative low concentrations of temazepam in the Wistar rats suggest a relatively reduced activity of the CYP3A isoenzymes compared to the Harlan–Fischer rats.

Similar differences in the pharmacokinetics of diazepam have been documented in mice (Griebel et al., 2000) and in Wistar rats (Van der Laan et al., 1993).

However, not only the hepatic/pharmacokinetic mechanisms may be the reason for observed variations in the efficacy of diazepam.

Another factor could be differences in the activity of central neurotransmission systems. There is considerable evidence of an important role for 5-HT in the control of anxiety (Chopin and Briley, 1987). It is established that an increased serotonergic activity combined with an increase in extracellular 5-HT is associated with anxiety, whereas a low extracellular 5-HT level is associated with an anxiolytic-like behaviour. In our study, the 5-HT levels were lower in the Harlan–Wistar compared to the 'anxious' Harlan–Fischer rats. These results correspond well with earlier studies, where a central application of 5-HT led to an anxiogenic-like behaviour (Wise et al., 1972) and nonselective 5-HT antagonists induced an anxiolytic-like behaviour (Cook and Sepinwall, 1975) and with a microdialysis study in our laboratory where an aversion-induced increase in 5-HT release on exposure to the X-maze was detected in Fischer rats but not in the Harlan–Wistar rats (Rex et al., 1999). Increased anxiety could be detected following a potentiated 5-HT release during the stay on the X-maze (Marsden et al., 1995).

.Inhibitory effects of benzodiazepines on central 5-HT synthesis, release and 5-HT turnover have been documented (Lister and File, 1983; Stein et al., 1973). Further on, in vivo experiments have demonstrated declined 5-HT release in awake animals (Pei et al., 1989; Soubrie et al., 1983) after systemic benzodiazepine injection. In our experiments, diazepam also decreased 5-HT levels in the two terminal regions.

The differences in the inhibitory effect of diazepam on the activity of the serotonergic system could also modify the anxiolytic effects of diazepam (Marsden et al., 1995).

The behavioural, neurochemical, as well as the pharmacokinetic, data indicate that genetic factors substantially contribute to the efficacy of anxiety-modulating drugs in different rat strains. The results demonstrate how the validity of an animal model of anxiety may be disputed because the results are sometimes not comparable using different rat strains. It is well established that children of parents with anxiety disorder are at high risk for anxiety disorder, indicating also a genetic predisposition (e.g., Skre et al., 2000). Therefore, knowledge of the genetic variations can provide a better view of drug effects and sensitivity useful to improving the efficacy of drug development and utilization in the treatment of anxiety. In conclusion, different strains in animal models of anxiety should be used with caution. Determination of the causes for distinct behaviour and the subsequent biochemical and possibly genetic differences may be an effective way to assess the neurochemical basis of anxiety-related behaviour.

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