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Comparison of Behavioral and Central BDZ Binding Profile in Three Rat Lines

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ONAIVI, E. S., P. A. MAGUIRE, N. F. TSAI, M. F. DAVIES AND G. H. LOEW. *Comparison of behavioral and central BDZ binding profile in three rat lines.* PHARMACOL BIOCHEM BEHAV 43(3) 825–831, 1992. – The performance of three widely used rat lines (Sprague-Dawley, Wistar, and Long Evans hooded) were evaluated in behavioral test systems that are sensitive to benzodiazepines. The in vivo effects of flunitrazepam and the brain [^{3}H]Ro 15-1788 binding were determined and compared in these rat lines. The behavioral end points evaluated in this study were anxiolysis, measured using the automated elevated plus-maze; sedation by modification of locomotor activity; hyperphagia following food deprivation; protection for pentylenetetrazol-induced convulsions; and hypothermia. There were comparable results in the hypnotic, hypothermic, anticonvulsant, and feeding tests in these lines following flunitrazepam administration. However, the behavior of the Long Evans hooded rat was most amenable to the detection of drug-induced changes in the anxiety test. There was no difference in the maximum number of binding sites (B_{max}) or the affinity (K_i) of the Ro 15-1788 or flunitrazepam binding in either the cerebellum or whole brain (minus cerebellum) in the three rat lines as determined by the competitive binding against [^{3}H]Ro 15-1788. Thus, while these rat lines exhibited similar behavioral profiles in most tests the modest differences in the baseline responses and the ability to detect anxiolysis at lower doses of flunitrazepam observed with Long Evans hooded rats makes them particularly suited for these types of studies.

Anxiety Behavior Benzodiazepines BDZ binding Body temperature Feeding
Rat lines Sedation

THE many behavioral properties of benzodiazepines (BDZs) such as anxiety reduction and induction, muscle relaxation, sedation, and anticonvulsant effects have been extensively studied over the years in numerous laboratories (15,30). Sprague-Dawley, Wistar, and Long Evans hooded rats are the most commonly used strains of rats in such studies for the screening and investigation of the mechanism of action of these compounds. The animal behavioral responses to drugs have been shown to differ in rat (5,7,10,14) and mice (21–24,38) lines. Strain differences in response to novel or stressful stimuli as an indicator of "emotionality" have been demonstrated after the administration of BDZs to rats (1,3,4,6,16). Neurochemical and BDZ receptor differences between Maudsley rat strains (1,2,18,25,31,33,37,39,40) have been reported. It has also been demonstrated that genetically determined traits play an important role in regulating the effects of abused drugs like cocaine, opiates, and ethanol (5,8,9,11–13,34,36).

Thus, for the purpose of validating animal behavioral test systems for the evaluation of BDZs the profile of the three rat lines in the anxiety, hypnotic, hypothermic, and hyperphagic tests were determined using a reference BDZ agonist, flunitrazepam. In parallel experiments, using the same three rat lines, the binding of Ro-15-1788 and flunitrazepam were determined by competitive binding against [^{3}H]Ro 15-1788 to ascertain whether the sensitivity of the rat lines to the effects of flunitrazepam could be correlated with the BDZ receptor density and affinity.

METHOD

Animals

Male rats of the Wistar, Sprague-Dawley, and Long Evans hooded lines weighing 250–500 g were used throughout this study. They were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed in fours (with rats of the same lines housed together). Animals were maintained on a reverse 12 L : 12 D cycle, with lights off by 6:00 a.m. Rats were adapted to this reverse light-dark cycle for at least 2 weeks prior to drug treatment and behavioral testing. Animals had free access to Purina Rat Chow and water at all times except when deprived of food prior to the ingestational behavior test. On test days, rats were transported from the dark holding room at 9:00 a.m. in a dark carrier to a room dimly illuminated with a 60-W red bulb. After 1 h of habituation to the new environment, drug or vehicle administration commenced, followed by behavioral testing.

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Experimental Design

The experimented protocol for the behavioral studies was divided into three groups of tests. The first group of tests consisted of the measurements of open-field activity, performance in the elevated plus-maze, and changes in rectal temperature in the same group of animals. Food consumption following food deprivation was determined in separate groups of animals, as was convulsant/anticonvulsant activity. The effect of IP administration of vehicle or flunitrazepam in a volume of 1.0 ml/kg weight was then evaluated in these tests. In parallel experiments, the binding of Ro 15-1788 and flunitrazepam to membranes prepared from the cerebellum and whole brain (without the cerebellum) were determined for these rat lines by competitive binding against [^{3}H]Ro 15-1788.

Behavioral Tests

Performance in the automated elevated plus-maze. The effect of flunitrazepam on the behavioral measures indicative of changes in anxiolytic- or anxiogenic-like profile were evaluated in the three lines using a computer-controlled elevated plus-maze test system. The apparatus consisted of two open arms (50 × 10 cm) and two enclosed arms (50 × 40 × 10 cm) that were linked by a central platform (10 × 10 cm) and arranged such that the arms of the same type were opposite each other. The apparatus was constructed of a dark vinyl Plexiglas material and mounted on a clear plastic base with a 50-cm elevation above the floor. The experimental procedures used were similar to those described by Pellow et al. (29) and according to the modification by Onaivi et al. (28). The test system was further altered by the addition of 12 pairs of infrared photocell units. The photocells and their receivers were located 3 and 5 cm above the test platform at the entrances to each of the open and closed arms and also at the diagonal medians of the central platform. The interruption of the photocell beams by the animals was monitored via an interface (D-max 54, Newark, NJ) connected to the IBM PC computer, which was located in an adjacent room away from the test arena. With this arrangement, the movement and location of the rat during a 5-min test was continuously displayed and monitored and recorded from a remote facility. The testing was initiated 30 min after vehicle or flunitrazepam administration by placing each animal in the center of the plus-maze facing an open arm. The number of entries and the amount of time spent in the open arms, closed arms, and center platform were recorded. The total time spent in the open and closed arms was usually less than 5 mins because the period of time spent on the central platform was not used in the data analysis.

Measurement of changes in rectal temperature. Animals were acclimatized to the laboratory condition where the ambient temperature of the room was maintained at 23 ± 2°C. Rectal temperatures were recorded using a rat rectal probe inserted 4 cm into the rectum (Digital Thermometer, Fisher Scientific, Fair Lawn, NJ), according to the method of Jackson and Nutt (18). Rectal temperatures were taken prior to injection of vehicle or flunitrazepam and again 30 min after treatment. The first reading was taken to familiarize animals with the experimental procedure and ensure that drug responses were not masked by the small hyperthermic response that occurs when animals are initially handled (41). Drug effects on temperature were measured 30 min after drug treatment.

Open-field activity test. In this study, the open-field activity test was adapted for the assessment of sedation and a general depressant action following administration of vehicle or flunitrazepam. The test was undertaken for 10 min immediately after testing in the elevated plus-maze. The open-field activity monitors consisted of circular test cages 46 cm in diameter and 34 cm high, each equipped with six pairs of photocells and detectors located diametrically opposite each other, 2 cm above the grid floor. Interruptions of the photocell beams were recorded automatically by digital counters.

Ingestional behavioral test. Feeding behavior in the rat lines were assessed following a 16-h period of food deprivation. Animals were habituated to eating rat chow pellets modified to make them more palatable. On a test day, groups of rats were injected with doses of flunitrazepam or its vehicle and placed in individual cages. Thirty minutes later, the test commenced with the introduction of a 40-g quantity of chow pellets placed in cups in the individual cages. The duration of the food consumption test was 1 h, following which the remaining food in the cup was weighed and the amount consumed recorded. During this test, water and the rat's standard chow were not available. Care was taken to collect and account for food spillage. Animals were visually observed for other signs that may interfere with ingestional behavior.

Anticonvulsant test (pentylenetetrazol-induced convulsions). The proconvulsant activity of pentylenetetrazol (PTZ) and the ability of flunitrazepam to counteract the effects of PTZ were evaluated. Rat line differences in response to PTZ were assessed by conducting preliminary studies based upon which isoeffective doses for each of the rat lines could then be chosen. The anticonvulsant activity of flunitrazepam was assessed by pretreating animals with flunitrazepam 30 min before challenge with the aforementioned isoeffective doses of PTZ. Animals were observed for a 30-min period immediately following administration of PTZ. The number of subjects that convulsed and the duration of these convulsions were recorded.

Receptor Binding Assay

The binding assay method was performed as previously described (44). Briefly, rats were killed by decapitation and their brains rapidly removed and frozen at −80°C. After thawing, the cerebellum was separated from the remainder of the brain. Both tissues were homogenized with a polytron homogenizer in 40 vol 50 mM Tris-HCl, pH 7.7, at 0°C and centrifuged at 20,000 × *g* for 10 min. Each pellet was rehomogenized and centrifuged twice, frozen, thawed, and washed an additional two items. The membranes were suspended in a tissue concentration of 4.5 mg wet wt/ml in the assay buffer: 50 mM Tris-HCl, pH 7.7 at 0°C. The binding assay was performed using these membranes (1 ml) incubated in triplicates with 0.5 nM [^{3}H]Ro 15-1788 and 15 concentrations of unlabeled Ro 15-1788 or flunitrazepam in a total volume of 2 ml for 90 min. Nonspecific binding was determined in the presence of 1 μM Ro 15-1788. The reaction was terminated by filtration (Brandel Cell Harvester, Gaithersburg, MD) through glass fiber filters (Whatman GF/B, Gaithersburg, MD) followed by 3.5-ml washes with ice-cold buffer. Radioactivity retained on the filters was determined by liquid scintillation with ReadySafe (Beckman Instruments, Fullerton, CA) after 12 h at room temperature. Protein assays were performed using the method of Lowry et al. (20).

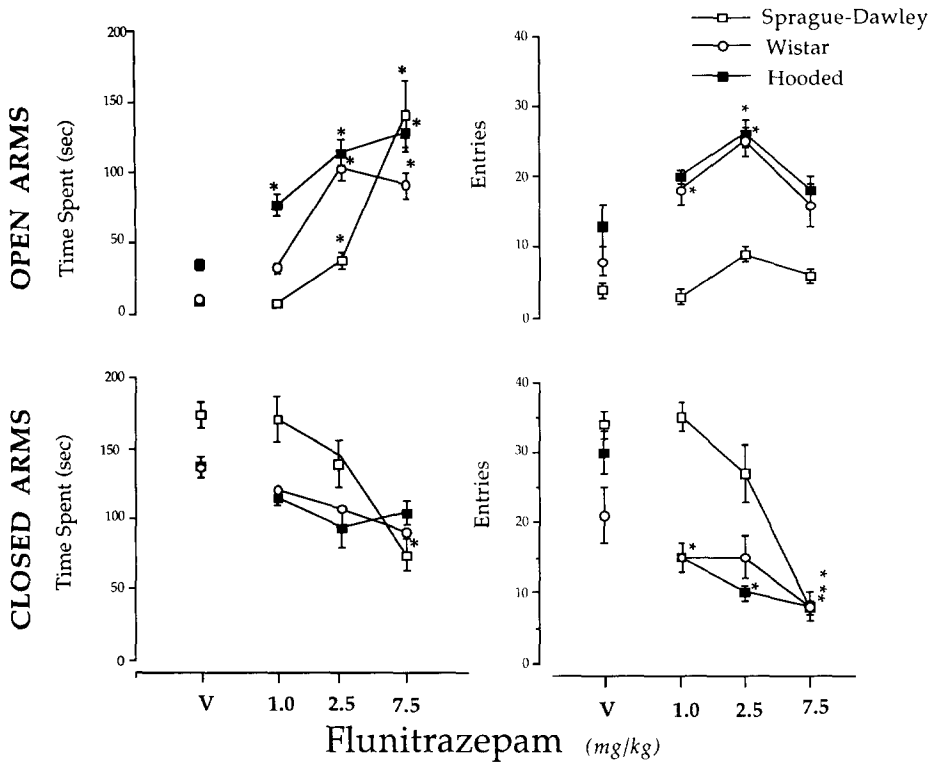

FIG. 1. Performance of the three rat lines and the effect of flunitrazepam in the elevated plus-maze test system. The time spent and the number of entries into the open and closed arms during the 5-min test session are shown. Flunitrazepam or its vehicle was administered 30 min prior to placement of the animal in the center of the plus-maze facing an open arm. Values are expressed as means ± SEM. The significant differences from vehicle-treated animals are indicated as $*p < 0.05$ (one-way ANOVA followed by Dunnett's t-test; n = 10–20).

Statistical Analysis

The behavioral results were analyzed using one-way analysis of variance (ANOVA) with multiple comparisons and the drug treatment as the independent factor. Dunnett's t-test was used to study treatment differences. The data obtained from the competitive binding assays were analyzed by a modified version (42) of the program LIGAND (26), which calculates the receptor binding affinities and capacities using weighted nonlinear, least-squares regression analysis. ED_{50}s were calculated by the method of Litchfield and Wilcoxon (19).

Drugs

Ro 15-1788 was received as a generous gift from Hoffman-La Roche (Nutley, NJ). [^{3}H]Ro 15-1788 was purchased from New England Nuclear (Boston, MA) and flunitrazepam from Sigma Chemical Co. (St. Louis, MO). For the in vivo tests, flunitrazepam was injected as a suspension in 40% β-cyclodextrin purchased from Research Biochemicals, Inc. (Natick, MA).

RESULTS

Influence of Flunitrazepam on the Performance of the Rat Lines in the Elevated Plus-Maze Test

In preliminary experiments, non-drug-treated naive rats from the different lines (n = 10 per group) demonstrated aversion to the open arms of the elevated plus-maze and entered more frequently into the closed arms. As the responses of vehicle-injected and nontreated rats were indistinguishable, the baseline activity for rats treated with vehicle are the controls shown in Fig. 1. In this test, an index of anxiolytic-like profile was indicated by a combination of the following activities of the animal in the elevated plus-maze tests: a) an increase in the amount of time spent in and/or number of entries into the open arms and b) a reduction in the amount of time spent in and/or number of entries into the closed arms. Vehicle-treated Hooded rats spent significantly more time in the open (Hooded, 45 ± 3; Sprague-Dawley, 11 ± 5; and Wistar, 25 ± 6 s) and also entered the open arms more frequently than Wistar and Sprague-Dawley rats, $F(2, 29) = 12.42$, $p < 0.05$. Hooded rats were also more sensitive to the anxiolytic-like effect induced by 1 mg/kg flunitrazepam, the lowest dose used, when compared to the other two lines. Only hooded rats had a significant increase in time spent in the open arms and a reduction in the number of entries into the closed arms, $F(11, 71) = 26.46$, $p < 0.05$. Increasing the dose of flunitrazepam to > 1.0 mg/kg, however, induced the characteristic anxiolytic-like response in all three rat lines.

Modification of Open-Field Activity by Flunitrazepam

Assessment of open-field activity showed that hooded rats were more active than either Sprague-Dawley or Wistar rats, $F(2, 17) = 5.0$, $p < 0.05$. As shown in Fig. 2, flunitrazepam

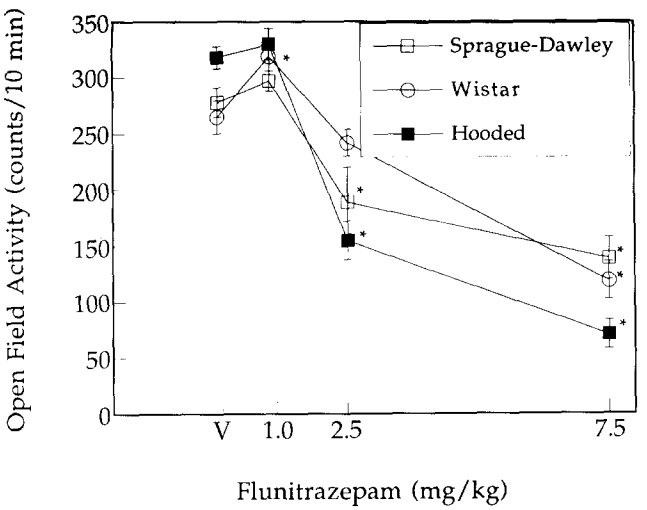

FIG. 2. Modification of locomotor activity as assessed in the open-field activity monitor equipped with photocells. Flunitrazepam was injected IP; then, rats were tested 30 min later immediately after the elevated plus-maze test. The duration of the test was 10 min. Values expressed are means ± SEM and significant changes in locomotor activity are indicated by $*p < 0.05$ (one-way ANOVA followed by Dunnett's t-test for multiple comparison with vehicle).

(1–7.5 mg/kg) significantly reduced open-field activity in the three rat lines with ED_{50} values of 4.5, 5.02, and 3.9 mg/kg in the Long Evans hooded, Wistar, and Sprague-Dawley rats, respectively. The 95% confidence limits overlapped; therefore, the ED_{50} values of the three rat lines were not significantly different.

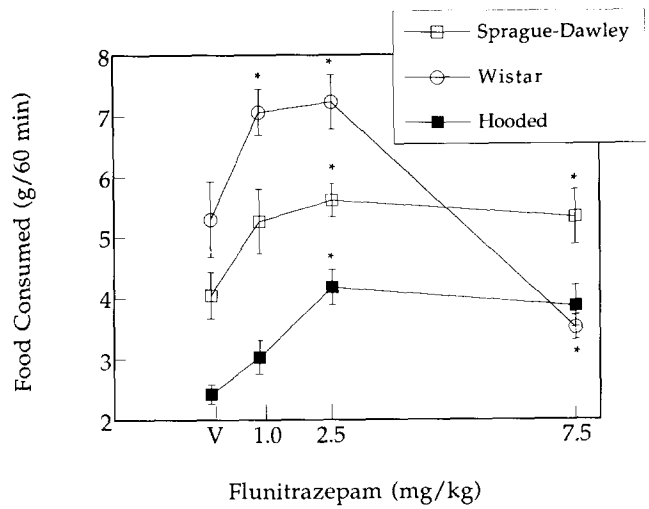

FIG. 3. Hyperphagic effect of flunitrazepam in the different rat lines. Feeding was increased by the 1.0- and 2.5-mg/kg doses while the higher doses were disruptive over the 60-min test period. The results are shown as mean food intake (g) ± SEM ($n = 10$ per group). Levels of significance are indicated as $*p < 0.05$ (one-way ANOVA followed by Dunnett's t-test for multiple comparison with vehicle).

Food Intake in the Different Rat Lines and the Influence of Flunitrazepam

Figure 3 illustrates the food intake of the different rat lines and the influence of flunitrazepam (1–7.5 mg/kg) on feeding behavior. At 1 mg/kg, flunitrazepam significantly increased feeding in Wistar rats, $F(3, 23) = 15.7$, $p < 0.05$, but not in hooded or Sprague-Dawley rats. The 2.5-mg/kg dose significantly increased feeding in all three rat lines, but a further increase in the dose of flunitrazepam to 7.5 mg/kg elicited sedation and decreased activity, thus disrupting feeding. In addition, although not explicitly quantified, stereotypic head-weaving was observed in Wistar rats at the highest dose.

Measurement of Changes in Rectal Temperature

The changes in rectal temperature following vehicle and flunitrazepam administration to the rat strains are presented in Fig. 4. With vehicle administration, there was a significant drop in rectal temperature in Wistar rats in comparison to Sprague-Dawley and Long Evans hooded rats, $F(2, 17) = 18.18$, $p < 0.05$. A dose-dependent drop in rectal temperatures was obtained in all the three rat lines following flunitrazepam treatment. Flunitrazepam-induced hypothermia was similar in these rat lines for the 1.0-mg/kg dose, $F(2, 17) = 1.523$, $p < 0.2499$; for the 2.5-mg/kg, $F(2, 17) = 0.17$, $p < 0.8449$; and for the 7.5-mg/kg dose, $F(2, 17) = 0.041$, $p < 0.9603$.

PTZ-Induced Convulsions and Anticonvulsant Effect of Flunitrazepam

Figure 5 shows the proconvulsive actions of PTZ in the three rat lines. The doses found to be isoeffective in the three rat lines were 60 mg/kg for Wistar and hooded rats and 52

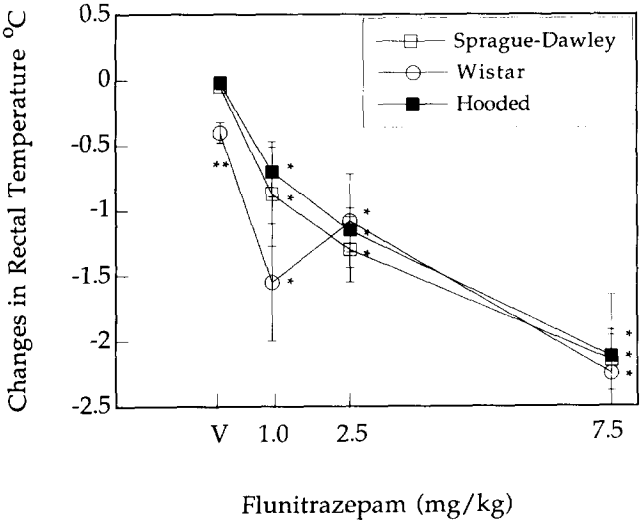

FIG. 4. Hypothermic effect of flunitrazepam in the different rat lines. The change in the temperature caused by vehicle is reported with respect to baseline values. The change in the temperature reported for each dose of flunitrazepam is reported with respect to the vehicle values. The results are shown as mean ± SEM ($n = 10$ per group). Levels of significance are indicated as $**p < 0.05$ for differences in the baseline values and $*p < 0.05$ for differences between drug and vehicle (one-way ANOVA followed by Dunnett's t-test for multiple comparison with vehicle).

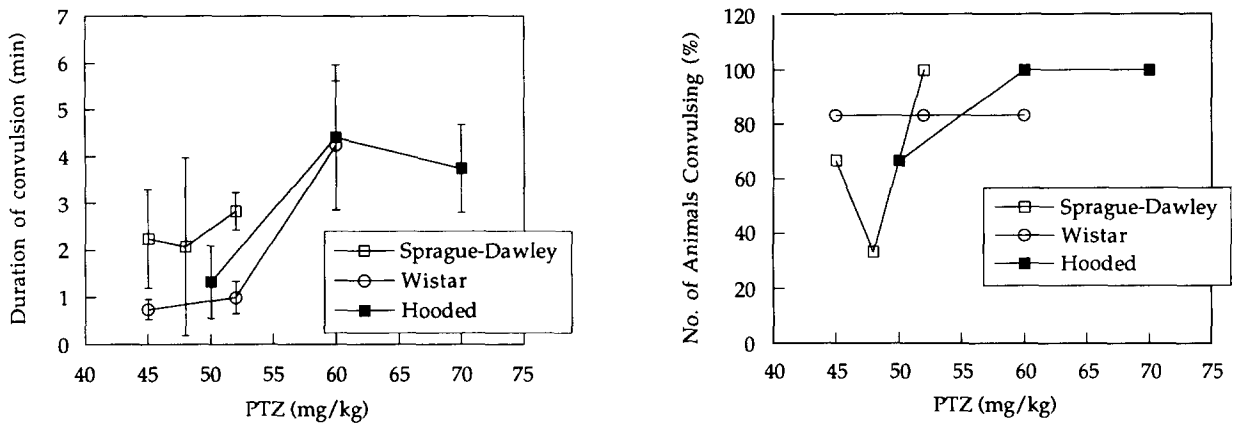

FIG. 5. Proconvulsant effect of PTZ in the three rat lines. The mean duration of convulsions ± SEM and the percentage of animals that convulsed are shown. The observation period for the convulsion tests was 30 min.

mg/kg for Sprague-Dawley rats. These doses were chosen on the basis of ability to reliably induce convulsions of maximal duration and minimal lethality.

Figure 6 shows that, in all three rat lines, flunitrazepam (0.01–0.2 mg/kg) decreased both the convulsion duration and the percentage of subjects experiencing convulsions induced by isoeffective doses of PTZ. The F values for the ANOVA in convulsion duration were, $F(4, 29) = 3.121$, $p < 0.0327$, for hooded rats; $F(4, 28) = 2.224$, $p < 0.0965$, for Sprague-Dawley rats; $F(4, 29) = 6.398$, $p < 0.0011$, for Wistar rats. There were no appreciable differences in the anticonvulsant activity of flunitrazepam between the three rat lines.

Competitive Binding Against [^{3}H]Ro 15-1788

A comparison of the receptor binding parameters, K_i and B_{max}, for the three strains of rats is shown in Table 1. The competitive binding assays were performed against [^{3}H]Ro 15-1788 with flunitrazepam and Ro 15-1788 as the competing ligands. The K_i and B_{max} values were calculated using LIGAND, and data from each tissue was best fit to a one-site model. The total number of binding sites were similar in each strain. The K_i for flunitrazepam and Ro 15-1788 were not different in the cerebellum and whole brain without the cerebellum in the three rat strains.

DISCUSSION

The major objective of this study was to determine the profile of three commonly used rat lines, with the intent of determining the appropriate rat line to use for the evaluation of BDZs in these behavioral tests. The Long Evans hooded rats appeared to be particularly suited for the evaluation of the BDZ-like compounds. In the elevated plus-maze test, in the absence of flunitrazepam these rats, when compared to Sprague-Dawley and Wistar rats, spent more time in the open arms of the maze. This characteristic would allow for better detection of possible anxiogenic drug effects. Of the three rat lines, hooded rats appeared to be most sensitive to the effects of the reference BDZ flunitrazepam in the plus-maze test. Unlike Wistar rats, hooded rats did not experience a significant decrease in body temperature with vehicle administration

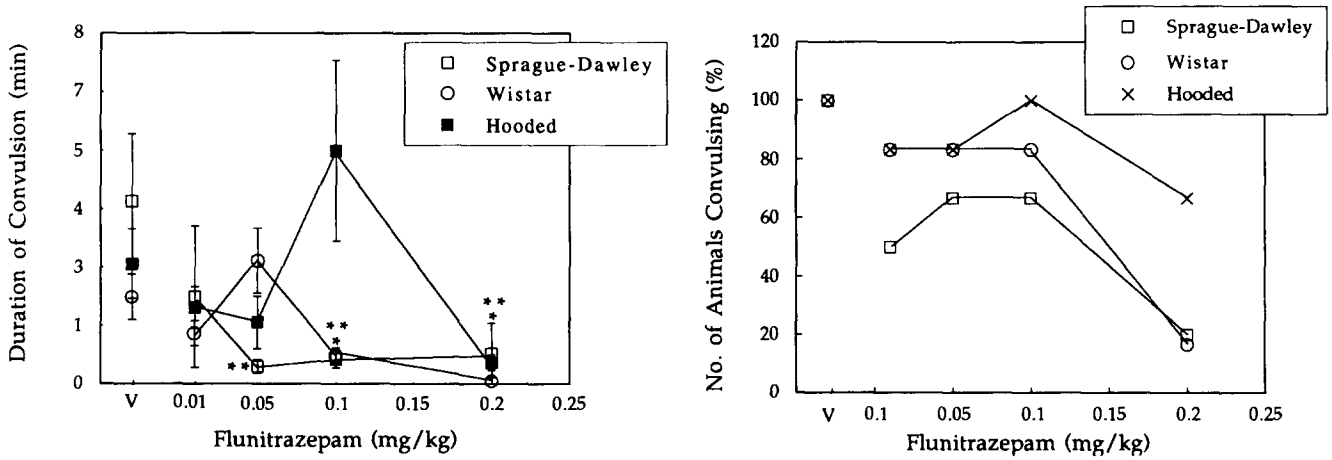

FIG. 6. Anticonvulsant effect of flunitrazepam in the three rat lines. Convulsion was induced by isoeffective doses of PTZ. Flunitrazepam was administered intraperitoneally 30 min before PTZ and the observation period of the test was 30 min. Significant changes from vehicle-treated animals are indicated as $*p < 0.05$ for Wistar rats and $**p < 0.05$ for Sprague-Dawley rats (one-way ANOVA followed by Dunnett's t-test).

TABLE 1

COMPARISON OF RECEPTOR BINDING PARAMETERS, K_i AND B_{MAX}, FOR THE THREE STRAINS OF RATS

	Sprague-Dawley	Wistar	Hooded
Flunitrazepam			
K_i, cerebellum (nM)	2.15 ± 0.08	2.60 ± 0.11	2.31 ± 0.09
K_i, whole brain (nM)	2.03 ± 0.05	2.43 ± 0.08	2.75 ± 0.19
Ro 15-1788			
K_i cerebellum (nM)	0.55 ± 0.03	0.67 ± 0.04	0.64 ± 0.03
K_i whole brain (nM)	0.63 ± 0.03	0.65 ± 0.03	0.85 ± 0.08
B_{max}, whole brain (fmol/mg protein)	703 ± 31	678 ± 41	815 ± 99

Competitive binding assays were performed, in triplicate, against [^{3}H]Ro 15-1788 in membranes prepared from cerebellum and whole brain (-cerebellum) of each rat strain. The K_i and B_{max} were calculated suing LIGAND. The values reported are mean ± SE.

or display head-weaving upon treatment with the highest dose of flunitrazepam. These behaviors could interfere with the behavioral tests.

Some studies have demonstrated differences in BDZ receptor binding in Roman rat strains (27,32,33,35). A correlation between benzodiazepine receptors and emotionality has been suggested because of the existence of fewer binding sites in Maudsley reactive rats, which show greater emotional reactivity than Maudsley nonreactive rats (4), although this has not been confirmed by others (40). However, the baseline differences in the rat lines and the differences obtained in the anxiety tests in this study could not be reliably correlated to the BDZ receptor binding as determined by the use of a nonselective BDZ ligand. It is to be noted that numerous subunits of the $GABA_A$ receptor-Cl^- channel complex comprising receptors with different affinities for GABA and BDZs have now been characterized (43) and furthermore that receptor populations may vary within smaller regions of the brain. The crude level of dissection and the use of the nonselective radioligand [^{3}H]Ro 15-1788 may have obscured the possibility of detecting significant differences in the BDZ receptor characteristics.

In summary, there are clear baseline differences in the behavioral responsiveness of Long Evans hooded rats in comparison to Wistar and Sprague-Dawley rats. An anxiolytic action of the lowest dose of flunitrazepam could be detected only in Long Evans hooded rats, although the drug produced comparable changes in the feeding, hypnotic, anticonvulsant, and hypothermic tests. The behavioral differences cannot be readily explained by the methods utilized in the receptor binding studies as there were no significant differences between rat lines in either receptor density or the ligand affinity at CNS BDZ sites.

ACKNOWLEDGEMENTS

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