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# Effects of repeated testing in two inbred strains on flesinoxan dose–response curves in three mouse models for anxiety

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**Abstract**

Over the last decade, many genetically modified mice have been developed as models for psychiatric diseases such as anxiety. Limited availability of such mutant mice highlights the importance of studying the possibility of repeatedly testing the same individuals. We tested mice four times with 1-week intervals with the same dose of the 5-$HT_{1A}$ receptor agonist flesinoxan (0–0.3–1.0–3.0 mg/kg s.c.) in three anxiety-related paradigms: light–dark exploration, open-field activity and stress-induced hyperthermia. The two inbred strains studied were the highly anxious 129S6/SvEvTac (S6) and low-anxiety C57BL/6J (B6) mice. The results indicate that the effects of repeated testing were relatively mild. B6 mice showed some mild habituation in the open-field test when treated with vehicle, whereas S6 mice developed reduced initial activity in the light–dark box after drug treatment. In contrast, responses to flesinoxan treatment were strong and highly consistent for most parameters. In the open-field and light–dark tests, B6 mice showed reduced activity and anxiogenic-like behavioral responses, whereas S6 mice were minimally affected. Anxiolytic-like responses were found in both strains in the stress-induced hyperthermia paradigm. We conclude that B6 and S6 mice can be tested repeatedly with agents such as 5-$HT_{1A}$ receptor agonists with 1-week intervals in the three paradigms tested.


*Keywords:* Physiology; Behavior; Animal model; Flesinoxan; Repeated testing; 5-$HT_{1A}$ receptor

## 1. Introduction

Over the last decade, the interest in mouse research strongly increased in response to the development of gene targeting techniques. Genetic manipulations are routinely performed on embryonic stem cells from a 129 substrain and mice are often backcrossed onto strains such as C57BL/6 (Simpson et al., 1997). Therefore, it is important to collect behavioral data on these two inbred strains per se (Crawley et al., 1997; Homanics et al., 1999), to better understand the significance of a particular mutant phenotype, and to avoid false positive results that might be due to the background effects instead of the targeted gene (Gerlai, 1996). It is well known that 129S6/SvEvTac (S6) and C57BL/6J (B6) mice show different responses in a variety of anxiety models. Based on behavioral paradigms like open-field activity, elevated plus and zero maze, and light–dark exploration, the 129 strains are labeled as 'high-anxiety' mice, whereas the C57BL/6 mice are labeled as 'low-anxiety' mice (Bouwknecht and Paylor, 2002; Homanics et al., 1999; McIlwain et al., 2001; Rodgers et al., 2002; Voikar et al., 2001). In addition, the level of locomotor activity is different between these two strains. C57BL/6 mice are far more active than the 129 strains (Logue et al., 1997; Paulus et al., 1999).

After years of successfully reducing the number of animals used in medical research according to the three R's (reduction, refinement and replacement) (Russell and Burch, 1959), the success of gene targeting techniques has led to an opposite trend (Van Zutphen, 2002). Typical pharmacolog-

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ical studies for instance require a large number of animals when naive mice are used. Moreover, most laboratories breed their own lines of different mutant mice, so space and number of mice per project becomes a limiting factor. Thus, there are multiple reasons to perform studies directed at reducing the number of animals used in a research project. In a recent paper, we described the consistency of strain differences in mice tested repeatedly in the light–dark and stress-induced hyperthermia paradigm without drug treatment with 1-week test intervals (Bouwknecht and Paylor, 2002). The present study determined whether responses to a drug treatment are also consistent with repeated testing. In the current study, we evaluated the responses of B6 and S6 mice on three different mouse assays measuring unconditioned forms of anxiety (e.g. reviews, Belzung and Griebel, 2001; Treit, 1985): the light–dark exploration test (Bourin and Hascoet, 2003; Costall et al., 1989; Crawley and Goodwin, 1980; Hascoet et al., 2001), the open-field activity test (Christmas and Maxwell, 1970; Prut and Belzung, 2003) and the stress-induced hyperthermia paradigm (Borsini et al., 1989; Olivier et al., 2003; Van der Heyden et al., 1997). The first two behavioral assays are based on the innate aversion of mice to brightly lit open spaces, whereas the stress-induced hyperthermia paradigm measures a physiological response to mild stress exposure.

As a pharmacological tool, we used the selective 5-$HT_{1A}$ receptor agonist flesinoxan (0–0.3–1.0–3.0 mg/kg s.c.) (Wouters et al., 1988), because 5-$HT_{1A}$ receptors have been linked to anxiety in both animal models and humans (Gingrich and Hen, 2001). In addition, there appears to be an interaction between 5-$HT_{1A}$ receptors and genetic background in mice in relation to anxiety-related responses (Groenink et al., 2003). Flesinoxan has anxiolytic-like characteristics in various paradigms (e.g. Fish et al., 2000; Li et al., 2001; Groenink et al., 1995; King et al., 1997) and a longer half-life than 8-hydroxy-2-(di-*n*-propyl-amino)-tetralin (8-OH-DPAT) (Perry and Fuller, 1989; Zuideveld et al., 2002a,b), which makes it a suitable tool in order to avoid interference with the stress of the injection effects per se. For this project, a 5-$HT_{1A}$ receptor agonist was preferred over alternative anxiolytic drugs, such as benzodiazepines, because the latter class has been shown to develop one-trial tolerance (File et al., 1990).

We hypothesized that mice can be repeatedly evaluated in drug studies using unconditioned assays for anxiety with 1-week inter-test intervals.

## 2. Materials and methods

### 2.1. Subjects and maintenance

All experiments were performed at the Faculty of Pharmaceutical Sciences, Utrecht University, The Netherlands. C57BL/6J mice (B6: 120 males) were obtained from Jackson Laboratory, Bar Harbor, ME, USA and 129S6/SvEvTac mice (S6: 120 males) came from Taconic, Germantown, NY, USA. Mice were 8–9 weeks old at the start of the experiment. Prior to testing, mice were handled repeatedly and allowed to acclimate. Animals were housed socially per strain in type 2 cages (22 × 16 × 14 cm, 5 mice/cage) with water and food available ad libitum. Lights were on between 6:00 a.m. and 6:00 p.m. Cages were cleaned and bottles were filled with fresh tap water twice a week. Mice were housed and tested in accordance with NIH policies on use of animals in research. Both, the Animal Protocol Review Committee at Baylor College of Medicine, Houston and the Animal Ethical Committee of the Faculty of Pharmaceutical Sciences of Utrecht University, have approved the testing procedures.

### 2.2. Experimental

#### 2.2.1. Open-field activity test

The open-field test is performed in an automated Plexiglas Digiscan optical animal activity system (42 × 42 × 30 cm, RXYZCM, Accuscan, Columbus, OH, USA). White noise (± 55 dB inside the box) was present continuously in the test room. Additional lighting of the test arena provided 700–800 lx. Locomotor activity was scored automatically by breaking infrared beams that were 2.5 cm apart from each other (16 beams/side). Two sets of beams were positioned at the bottom of each side of the box to measure two-dimensional (*X*–*Y*) locomotion, whereas two additional, elevated rows registered vertical activity of the mouse. The software virtually separated the surface of the box in a center (25 × 25 cm) and an outer area by grouping subsets of beams. Mice were transported from the holding room to the test room 45 min prior to the first injection. Mice were injected and housed singly in a clean holding cage 70 min prior to the test, which was performed between 9:00 a.m. and 2:45 p.m. Placing the subject in the center of the open-field arena started the 30-min test. After the test, mice were put back into social housing in their original home cages.

#### 2.2.2. Light–dark exploration test

The light–dark test is a behavioral test for anxiety-related responses that is performed in a polypropylene box (44 × 21 × 21 cm) unequally divided in two chambers of which one is dark (1/3 of total surface) and the other area (2/3) is brightly lit (± 700–800 lx) (Crawley and Goodwin, 1980). A small opening (8.5 × 5 cm) allows mice to freely move from the illuminated to the dark chamber and vice versa. White noise (± 55 dB inside the box) was present continuously in the test room. Behavior was scored in detail with a hand-held computer (Psion Workabout mx, Psion Teklogix, Erlanger, KY, USA) using The Observer® (Noldus Information Technologies, Leesburg, VA, USA). The position of the mouse inside the box was scored as either being in the "dark," "light" or "far light" area (Bouwknecht and Paylor, 2002). Hereto, a line separated the illuminated chamber in two parts: one close to the

opening (light) and one further away (far light). As a result, all areas were equally sized. In order to score a transition from one area to another all four paws had to cross the line. Mice were transported from the holding room to the test room 45 min prior to the first injection. Mice were injected and housed singly in a clean holding cage 60 min prior to the test, which was performed between 9.00 a.m. and 2.15 p.m. The 10-min test started by placing a mouse in the light chamber at the opposite end facing the entrance to the dark chamber. After the test, mice were put back into social housing in their original home cages.

#### 2.2.3. Stress-induced hyperthermia paradigm

The stress-induced hyperthermia paradigm reflects a physiological response to mild stress exposure and is sensitive to treatment with anxiolytic drugs (Van der Heyden et al., 1997). In this procedure, mice were housed singly in the afternoon prior to the test in a clean type 2 cage with food and water ad libitum. On the test day, the experimenter entered the room 5 min before the first rectal temperature was determined ($T_1$: basal temperature). The probe (Thermalert TH-5, Physitemp, Clifton, NJ, USA) was dipped in oil and inserted in the rectum for about 2 cm. The mild stress of handling and probe insertion causes a hyperthermic response, which is determined 10 min later ($T_2$: stressed temperature). The difference ($\Delta T = T_2 - T_1$) reflects the stress-induced hyperthermia response. After the test, mice were put back into social housing in their original home cages. Mice were injected 60 min prior to the test, which took place between 9:00 and 11:00 a.m.

### 2.3. Experimental setup

The experiment was performed as a between-subjects design for the factor dose, where each subject was tested four times with the same dose of flesinoxan (0.0–0.3–1.0 or 3.0 mg/kg s.c. in saline; volume 10 ml/kg) in either the open-field, light–dark, or stress-induced hyperthermia paradigm ($n = 10$ per group). The order in which mice were tested was balanced. Using this design, the exact same dose–response curve was determined four times with 1-week intervals in order to determine the effects of repeated testing.

### 2.4. Data analysis

The present study primarily focuses on the consistency of drug responses in three anxiety-related paradigms for two inbred strains of mice. The differences between the two strains and the effects of the drug flesinoxan per se are secondary questions, which are only briefly discussed. A parallel study using a within-subject design for flesinoxan treatment focuses on the effects of drug and strain (Bouwknecht et al., 2004).

Data were analyzed in two different ways. Each parameter was determined repeatedly within the same subject. First, Pearson correlations were analyzed for naive (test 1) versus experienced (tests 2, 3 and 4) responses in the same individuals to determine the consistency independent of the drug treatment. Second, data were analyzed using a repeated measures analysis of variance (ANOVA: SPSS, Version 10 for Windows, Chicago, IL, USA) to determine the interaction and main effects for test and dose across all tests. Dose of flesinoxan was analyzed as a between-subjects factor and test number (1–4) as a within-subject factor for each strain and paradigm separately. A Greenhouse–Geisser correction was used to correct for putative violations of the sphericity assumption (Vasey and Thayer, 1987). When appropriate, post-hoc *t*-tests were performed with Bonferroni corrections to adjust for repeated comparisons.

For the open-field test, we focused on the following parameters: time and distance traversed in the center area, total distance in entire box and the frequency of vertical movements. In addition, the ratio of center divided by total distance was calculated as an anxiety-related measure. In the light–dark test, number of light–dark transitions, time spent in the dark area, latency to enter and rearing frequency was analyzed. For the stress-induced hyperthermia procedure, both temperatures ($T_1$ and $T_2$) were analyzed as a repeated measure as well as the stress-induced hyperthermia amplitude ($\Delta T = T_2 - T_1$).

## 3. Results

### 3.1. Open-field activity test

The indicator for anxiety in the open-field test, i.e. center/total distance showed highly significant correlations between the first naive and each of the retests in B6 mice, but not in S6 mice (Table 1). Overall absolute levels across all tests show that responses were rather consistent (Fig. 1). For both strains, there was no effect of repeated testing and no significant interaction between dose and repeated testing. B6 mice explored the center area of the box more than S6 mice. The activity in the center area was reduced in flesinoxan-treated B6 mice ($F_{3,108} = 40.57$, $P < 0.001$), and altered in a significant, non-linear way in S6 mice ($F_{3,108} = 9.79$, $P < 0.001$). In contrast to the linear dose-dependent effects in B6 mice, S6 mice showed increased center activity after the lowest dose (0.3 mg/kg).

The time spent in the center area is a second measure for anxiety. The correlation between naive and retests is highly significant for B6, but not for S6 mice. There was a significant interaction between drug and repeated testing in B6 mice ($F_{3,36} = 2.43$, $P < 0.05$) indicating that the effect of repeated testing was dose-dependent. Flesinoxan significantly reduced the time spent in the center area in B6 mice ($F_{3,36} = 24.46$, $P < 0.001$). In contrast, flesinoxan-treated S6 mice showed a trend ($F_{3,36} = 2.76$, $P = 0.06$)

Table 1
Pearson correlation across repeated tests in C57BL/6J (B6) and 129S6/SvEvTac (S6) mice

| Paradigm | Measure | Strain | Naive vs. test 2 | Naive vs. test 3 | Naive vs. test 4 |
|---|---|---|---|---|---|
| Open-field activity | Center/tot. distance | B6 | 0.741[a] | 0.742[a] | 0.686[a] |
| | | S6 | 0.101 | 0.103 | 0.063 |
| | Center time | B6 | 0.629[a] | 0.480[b] | 0.582[a] |
| | | S6 | −0.066 | 0.066 | 0.034 |
| | Total distance | B6 | 0.618[a] | 0.560[a] | 0.679[a] |
| | | S6 | 0.343[c] | 0.450[b] | 0.444[b] |
| | Center distance | B6 | 0.833[a] | 0.799[a] | 0.810[a] |
| | | S6 | 0.191 | 0.082 | 0.119 |
| | Vertical activity | B6 | 0.880[a] | 0.818[a] | 0.812[a] |
| | | S6 | 0.799[a] | 0.770[a] | 0.700[a] |
| Light–dark exploration | Transitions | B6 | 0.711[a] | 0.701[a] | 0.683[a] |
| | | S6 | 0.385[c] | 0.375[c] | 0.267 |
| | Time in dark | B6 | 0.731[a] | 0.716[a] | 0.469[b] |
| | | S6 | 0.476[b] | 0.437[b] | 0.258 |
| | Time to dark | B6 | 0.791[a] | 0.792[a] | 0.534[a] |
| | | S6 | 0.589[a] | 0.512[b] | 0.373[c] |
| | Rearing | B6 | 0.651[a] | 0.571[a] | 0.469[b] |
| | | S6 | 0.386[c] | 0.290 | 0.218 |
| Stress-induced hyperthermia | Amplitude | B6 | 0.595[a] | 0.632[a] | 0.639[c] |
| | | S6 | 0.499[b] | 0.492[b] | 0.424[b] |
| | Basal temp. | B6 | 0.470[b] | 0.633[a] | 0.525[a] |
| | | S6 | 0.695[a] | 0.516[b] | 0.711[a] |

Each subject was tested repeatedly at 1-week intervals with the same dose of flesinoxan (0.0, 0.3, 1.0 or 3.0 mg/kg s.c.) in either the open-field activity, light–dark exploration or stress-induced hyperthermia paradigm. Data presented are the correlation ($r$) between the first (naive) and each respective repeated test. Each correlation includes 40 mice for each strain (10 per dose of flesinoxan).

[a] Significance: $P<0.001$.

[b] Significance: $P<0.01$.

[c] Significance: $P<0.05$.

toward spending more time in the center area. This effect in S6 mice appears to reflect an initial reduction in locomotor activity in some animals, as reflected by the increased variance. That is, a portion of the flesinoxan-treated S6 mice tended to take longer to leave the initial start point in the center of the arena. The overall reduction in B6 mice suggests an anxiogenic effect of flesinoxan ($F_{3,36}=3.46$, $P<0.001$).

The total distance traversed in the open-field box is a general measure for locomotor activity. The correlation between naive and retested mice was significant for both strains for all comparisons. The dose × repeated testing interaction was significant for B6 mice ($F_{9,108}=2.20$, $P<0.05$), whereas both B6 and S6 mice showed main effects of repeated testing and dose (B6: $F_{3,108}=10.18$, $P<0.001$, $\varepsilon=0.79$ and $F_{3,36}=9.02$, $P<0.001$; S6: $F_{3,108}=5.81$, $P<0.01$, $\varepsilon=0.61$ and $F_{3,36}=5.37$, $P<0.005$, respectively). Overall, there was a slight decrease in locomotor activity in both strains when mice were tested repeatedly.

Similar to the center/total distance ratio data, there was a strong correlation across tests in B6 mice, but not in S6 mice for the distance traveled in the center of the arena. Only B6 mice showed a mild, but significant overall effect of repeated testing ($F_{3,108}=3.42$, $P<0.05$, $\varepsilon=0.86$), whereas flesinoxan caused a strong reduction in the distance traversed in the center in B6 mice ($F_{3,36}=5.81$, $P<0.001$) and a slight increase in S6 at a low dose only (0.3 mg/kg) ($F_{3,36}=8.26$, $P<0.001$). The pattern for the distance in center is rather similar to the effects seen for total distance, although the relative effect of flesinoxan is stronger on the center measure, which leads to a decreased center/total distance ratio seen in B6 mice.

Finally, the highly significant correlation for the vertical activity measure across multiple tests in both B6 and S6 mice indicates that this measure is quite consistent across multiple tests. The drug × repeated test interaction was significant for B6 mice ($F_{3,108}=3.46$, $P<0.01$) only. Although S6 mice showed minimal vertical activity, the effects of flesinoxan were dose-dependent ($F_{3,36}=4.70$, $P<0.01$) as a result of some activity at low, but not at high doses. Flesinoxan completely abolished vertical activity in B6 mice ($F_{3,36}=41.36$, $P<0.001$).

### 3.1.1. Light–dark exploration test

In the light–dark box, the number of light–dark transitions is the most commonly used parameter for assessing anxiety-related responses in mice. The correlation for this parameter was significant for some but not all retests in S6 mice, whereas B6 mice show very consistent responses (Table 1). Both strains show a slight reduction in light–dark transitions when tested repeatedly (Fig. 2; B6: $F_{3,108}=3.46$, $P<0.05$, $\varepsilon=0.74$; S6: $F_{3,108}=10.73$, $P<0.001$, $\varepsilon=0.53$). In S6 mice, transitions were low to start with and appeared to go down to almost one transition per test, which means that they move from the light into the dark without coming back out to explore the illuminated environment. Consistent with the literature, the 'low-anxiety' B6 strain showed more transitions than the 'high-anxiety' S6 strain. Flesinoxan severely reduced light–dark transitions in B6 ($F_{3,36}=33.23$, $P<0.001$), but not in S6 mice ($F_{3,36}=0.83$, $P=0.49$).

The second measure for anxiety is time spent in the dark area. The correlations between naive and retested mice were highly significant in B6 mice and for the first two, but not the third retest in the S6 mice. A significant effect of repeated testing was seen in both strains (B6: $F_{3,108}=3.97$, $P<0.05$, $\varepsilon=0.68$; S6: $F_{3,108}=3.57$, $P<0.05$, $\varepsilon=0.81$). The dose × repeated test interaction reached significance in S6 ($F_{9,108}=2.38$, $P<0.05$, $\varepsilon=0.81$), but not in B6 mice. S6 mice showed a mild increase after vehicle, but a decrease after flesinoxan when tested repeatedly. Overall, flesinoxan significantly decreased the

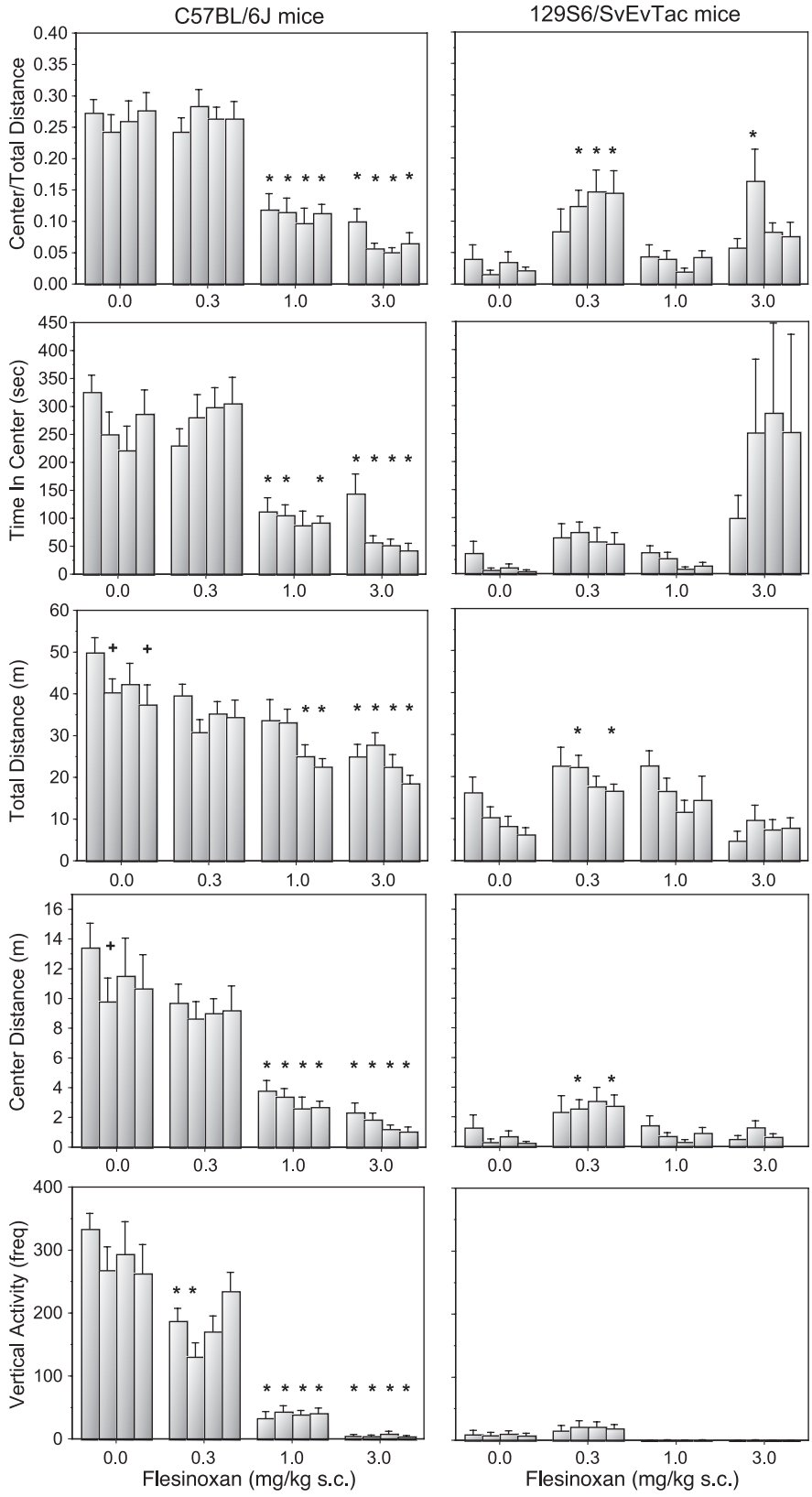


Fig. 1. Effects of flesinoxan and repeated testing in C57BL/6J (B6) and 129S6/SvEvTac (S6) mice in the open-field activity test. Mice ($n = 10$ per dose) were tested in a between-subjects design across doses. Adjacent bars represent mean ± S.E.M. for test 1, 2, 3 and 4, respectively, for each dose in the same animals (within-subject design); symbol: * means significantly different from saline treatment for the same test; + means significantly different from the first naive test. For detailed description of statistical analysis and overall effects, see Results section.

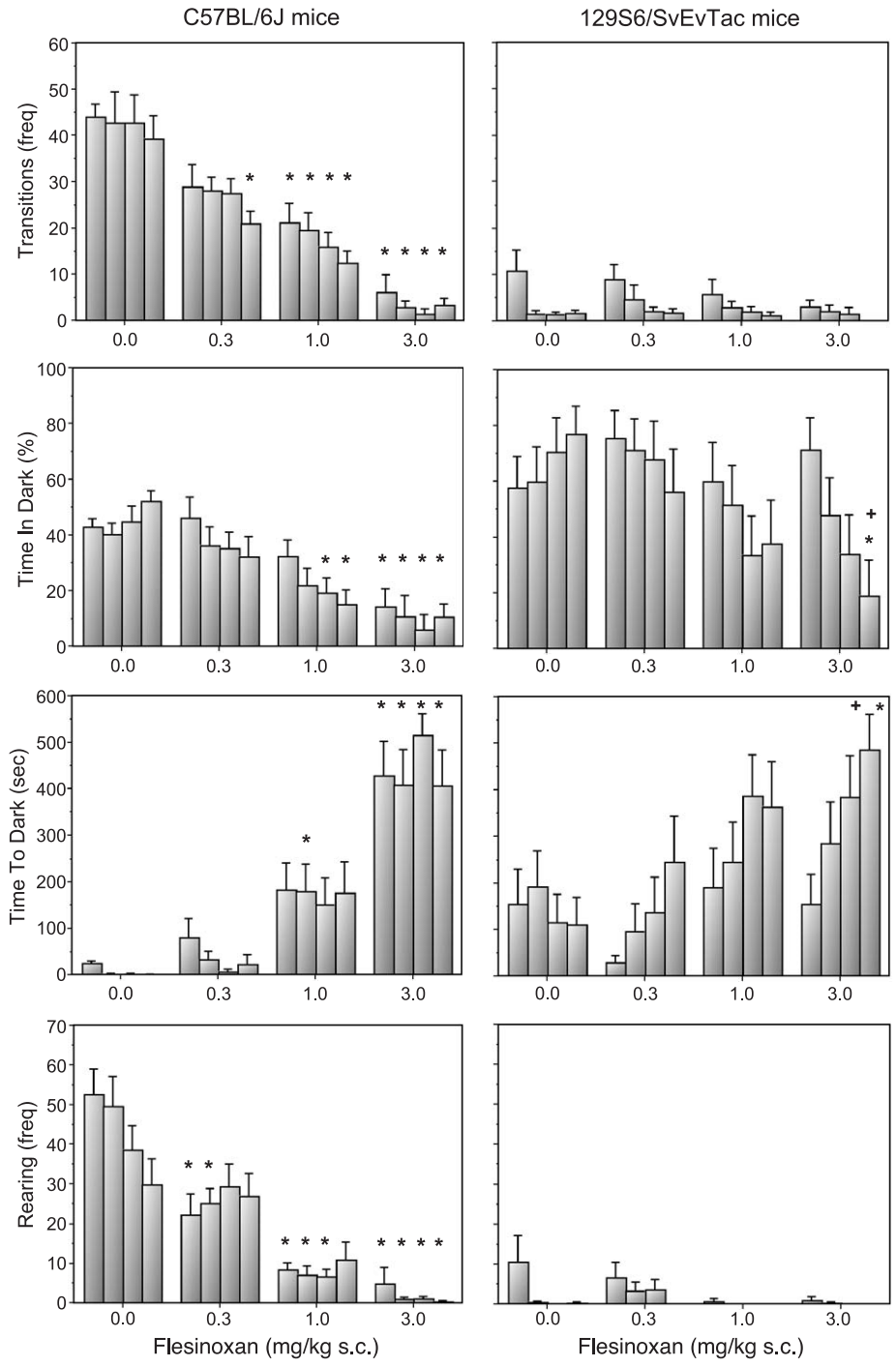


Fig. 2. Effects of flesinoxan and repeated testing in C57BL/6J (B6) and 129S6/SvEvTac (S6) mice in the light–dark exploration test. Mice ($n = 10$ per dose) were tested in a between-subjects design across doses. Adjacent bars represent mean ± S.E.M. for test 1, 2, 3 and 4, respectively, for each dose in the same animals (within-subject design); symbol: * means significantly different from saline treatment for the same test; + means significantly different from the first naive test. For detailed description of statistical analysis and overall effects, see Results section.

time spent in the dark in B6 mice ($F_{3,36} = 10.48$, $P < 0.001$).

The initial activity in the light–dark box is determined as the latency to move from the light into the dark. This parameter showed significant correlations between naive and retested responses in both strains. There was a significant drug × repeated test interaction for S6 mice ($F_{9,108} = 2.25$, $P < 0.05$, $\varepsilon = 0.80$). Repeated testing after vehicle injection had no effect on the response in S6 mice, whereas the latency increased when tested repeatedly after flesinoxan. In general, S6 took longer to enter the dark than B6 mice. Flesinoxan significantly increased this latency in B6 mice ($F_{3,36} = 31.75$, $P < 0.001$), whereas repeated testing had no effect.

Finally, as seen for vertical activity in the open-field paradigm, the correlation for rearing in the light–dark box was highly significant in naive versus retested animals for both strains. The interaction of dose × repeated testing was significant in B6 mice ($F_{9,108}=2.72$, $P<0.05$, $\varepsilon=0.64$) and flesinoxan severely reduced rearing ($F_{3,36}=33.25$, $P<0.001$). After vehicle injection, rearing slightly decreased across tests, whereas the lower levels were stable after flesinoxan treatment. Similar to the open-field data, S6 mice showed less vertical activity than B6 mice. The low levels in S6 mice were actually slightly reduced when mice were tested repeatedly ($F_{3,108}=4.70$, $P<0.05$, $\varepsilon=0.41$).

### 3.1.2. Stress-induced hyperthermia

In the stress-induced hyperthermia paradigm, the measure for anxiety is the increase in temperature over a 10-min window in response to the mild stress of measuring rectal temperature. We analyzed the stress-induced hyperthermia response and the basal temperature ($T_1$) after flesinoxan treatment.

The correlation between naive and retested mice was significant in both strains (Table 1).

No effect of repeated testing was found in B6 mice (Fig. 3). S6 mice showed a mild, but significant interaction between repeated testing and dose ($F_{3,108}=2.70$, $P<0.05$, $\varepsilon=0.85$), which appears to be caused by the unexpected decrease after 1.0 mg/kg during the last test. The stress-induced hyperthermia amplitude was significantly reduced by flesinoxan in both B6 and S6 mice ($F_{3,36}=48.86$, $P<0.001$ and $F_{3,108}=15.43$, $P<0.001$, respectively).

Basal temperature ($T_1$) was highly consistent in both strains as shown by the significant correlations between naive and retested mice (Table 1). Neither repeated testing nor the interaction with flesinoxan showed significant effects on $T_1$. Flesinoxan caused hypothermia in both strains (B6: $F_{3,36}=10.04$, $P<0.001$; S6: $F_{3,36}=30.66$, $P<0.001$). In B6 mice, all doses of flesinoxan reduced temperature to a similar level, while S6 mice showed a more dose-dependent response.

Overall, the stress-induced hyperthermia response showed more sensitivity to the various doses of flesinoxan in the B6 mice, whereas S6 mice were more sensitive to the flesinoxan-induced hypothermia.

## 4. Discussion

The development of genetically modified mice has reversed the annual decrease in number of animals used in animal experiments (Van Zutphen, 2002). In addition, it is

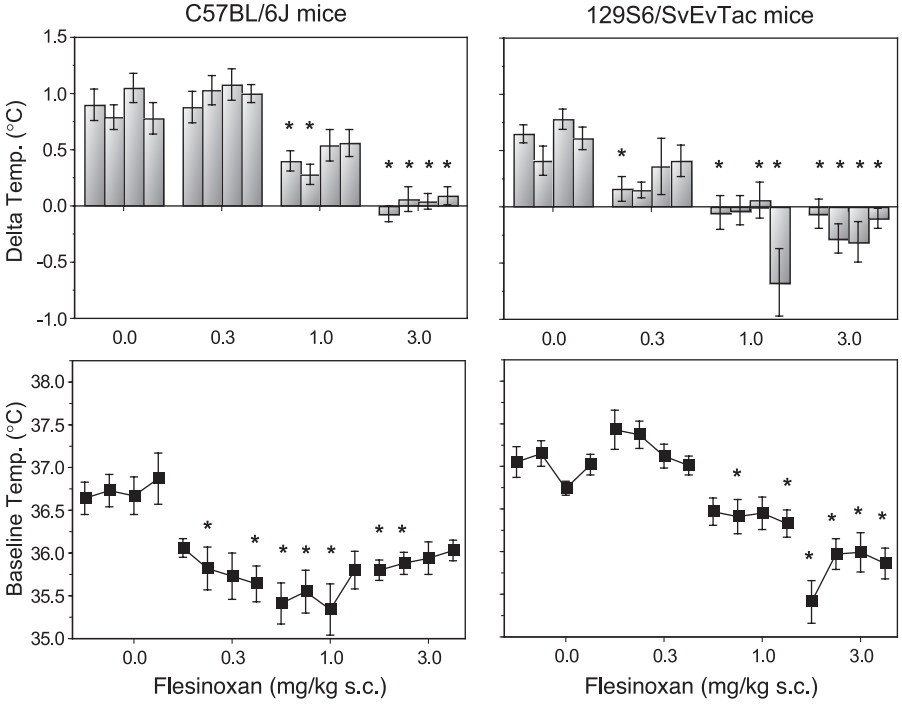


Fig. 3. Effects of flesinoxan and repeated testing in C57BL/6J (B6) and 129S6/SvEvTac (S6) mice in the stress-induced hyperthermia paradigm. Mice ($n=10$ per dose) were tested in a between-subjects design across doses. Adjacent bars represent mean ± S.E.M. for test 1, 2, 3 and 4, respectively, for each dose in the same animals (within-subject design). Markers connected by a line represent test 1, 2, 3 and 4, respectively, for each dose (within-subject design). The basal temperature ($T_1$) was determined 60 min after flesinoxan treatment, whereas $\Delta T$ represents the difference between basal and stressed temperature (determined 10 min after $T_1$ measurement). Symbol: * means significantly different from saline treatment for the same test; + means significantly different from the first naive test. For detailed description of statistical analysis and overall effects, see Results section.

often difficult to readily obtain enough mutant and wild-type mice for experiments that typically require large numbers of mice such as generating dose response curves for pharmacological studies. It is therefore important to determine whether mice can be tested repeatedly to potentially reduce the number of animals required for this type of research. We have recently performed studies to address this issue by using mice repeatedly in a test battery with multiple paradigms (McIlwain et al., 2001), and examining the consistency of differences between nine strains of mice which were repeatedly tested in two assays of anxiety-related responses (Bouwknecht and Paylor, 2002). To our knowledge, the present study represents the first attempt to directly assess the consistency of behavioral and physiological effects of a drug in two strains of mice tested repeatedly on the same paradigm. Each subject was treated with one dose of the 5-$HT_{1A}$ receptor agonist flesinoxan and tested four times with 1-week intervals in one out of three anxiety-related paradigms. These paradigms are all based on unconditioned responses, i.e. open-field activity, light–dark exploration and stress-induced hyperthermia. The study was performed in a so-called 'high- and low-anxiety' inbred strain (129S6/SvEvTac (S6) and C57BL/6J (B6) mice, respectively).

Our data analysis focused on anxiety-related parameters as well as several locomotor-related parameters. The anxiety-related measures are center/total distance (open-field paradigm), time in center (open-field), transitions (light–dark), time in dark area (light–dark) and $\Delta T$ (stress-induced hyperthermia).

Significant Pearson correlations indicate that the response distribution across individuals is consistent, whereas the absolute levels can be different. The two selected strains showed important differences at multiple parameters. In B6 mice, every single correlation between naive versus retested data sets was significant. So, the individual differences were consistent across multiple tests in B6 mice. For S6 mice, in contrast, correlations for several anxiety measures in the open-field and light–dark paradigms were not significant. Such a lack of significant correlations is partly related to the low activity levels in S6 mice, which leads to floor effects with limited variation. The physiological stress-induced hyperthermia is locomotion independent and showed highly significant correlations for the amplitude in B6 and S6 mice.

In addition to the correlations, ANOVA analyses were performed to study variance patterns across the complete data set including all four tests. When appropriate, post-hoc comparisons determine whether the absolute mean between two selected subsets of data is significantly different. Although some overall significant effects of repeated testing and interactions between dose and repeated testing were found, post-hoc comparisons revealed only a very limited amount of significant effects of repeated testing in B6 (open-field test) and S6 mice (light–dark test). For example, after 3.0 mg/kg flesinoxan, S6 mice spent less time in the dark area (light–dark) in test four compared to the first test. This effect could be explained, however, by reduced initial activity seen as a severe increase in latency to move from the starting point in the 'far-light' into the dark area of the light–dark box. Repeated testing had no effect in the stress-induced hyperthermia paradigm. Thus, the effects of repeated testing were rather limited on the anxiety measures.

For locomotor activity parameters, only saline-treated B6 mice showed some mild but significant habituation in the open-field test for total and center distance, as described before (Cook et al., 2002). In S6 mice, the only indication of habituation in response to repeated testing was the increase in latency to enter the dark area of the light–dark box. These habituation effects were rather mild compared to the dramatic drug effects on both horizontal and vertical components of locomotor activity in the open-field and light–dark test in B6 mice. Our data confirm previous findings with other 5-$HT_{1A}$ receptor agonists in rats and mice (Ahlenius et al., 1993; Blanchard et al., 1997).

The focus of the present study was to determine if drug-induced changes in anxiety-related responses changed with repeated dosing/testing. The effects of flesinoxan were very consistent affecting both anxiety-related and locomotor-related parameters. We found similar effects in a parallel study (Bouwknecht et al., 2004), designed to focus on dose–response curves for flesinoxan and comparing the same two genotypes. In the present study, flesinoxan reduced the center/total distance ratio and time in center in the open-field paradigm in B6 mice, while the low dose induced an increased center ratio in S6 mice and had no significant effect on time in center. The latter parameter showed large variance for the higher dose, which is probably related to reduced activity in some but not all S6 mice. In the light–dark box, flesinoxan reduced transitions and time spent in the dark in B6 mice and again had no effect in S6 mice. Each mouse started in the center of the open-field or the far-light area of the light–dark box, which seems to influence some of the anxiety parameters when the drug of interest has a direct effect on locomotion. Only the lowest dose of flesinoxan suggested an anxiolytic effect in S6 mice in the open-field test. Opposite results from low versus high doses of 5-$HT_{1A}$ receptor agonist has been described before (Misslin et al., 1990; Kilfoil et al., 1989). The stress-induced hyperthermia paradigm, in contrast, is locomotion insensitive and mice are tested in a familiar environment. Flesinoxan dose-dependently reduced the hyperthermic response in both B6 and S6 mice confirming previous findings (Bouwknecht et al., 2000; Olivier et al., 1998).

So, locomotion-related parameters were affected by flesinoxan as seen by decreased activity in the open-field in B6 mice, and increased latency to enter the dark area of the light–dark box. The lowest dose of flesinoxan induced a small increase in locomotion in S6 mice throughout the open-field box, but higher doses had no significant effect. Finally, flesinoxan not only reduced the stress-induced hyperthermia amplitude, but also caused hypothermia in

both inbred strains mice, which is consistent with previous findings (Bouwknecht et al., 2000; Olivier et al., 1998).

It is difficult to compare our data, which clearly demonstrates a rather mild effect of testing mice repeatedly with 1-week intervals, with the existing literature because similar designs have not been employed. While performance in rats on an elevated-plus maze is stable without drug treatment, benzodiazepine treatment during the first test induces so-called one-trial tolerance (File et al., 1990). DBA/2 mice show habituation after pre-exposure to the elevated-plus maze and increased exploration in the light–dark box (Rodgers and Shepherd, 1993), however daily treatment with benzodiazepines reduced anxiety measures in elevated-plus maze and light–dark test in test-naive mice, but not in maze-experienced mice (Rodgers and Shepherd, 1993). Finally, drug-free pre-exposure to the light–dark box, 24 h before the test blocks the anxiolytic effects of benzodiazepines (Holmes and Rodgers, 2003). These studies suggest that there may be limited effects of repeated testing with drugs acting on the $GABA_A$-benzodiazepine system.

It appears that an important factor in retesting subjects is the inter-test interval length. Daily testing of mice in the stress-induced hyperthermia paradigm increases basal temperature (Van der Heyden et al., 1997), severely reduces exploration in the light–dark box (Onaivi and Martin, 1989) and open-field test (Cook et al., 2002). Daily testing of rats with anxiolytic drug treatment in the open-field paradigm affects multiple parameters such as peripheral movements, rearing and grooming (Angrini et al., 1998). We have recently shown, however, that drug-free 1-week intervals, as used in the present study, results in consistent responses in the light–dark and stress-induced hyperthermia paradigm across multiple inbred strains (Bouwknecht and Paylor, 2002).

In summary, we conclude that the effects of repeated testing at 1-week intervals in inbred strains such as B6 and S6 mice in unconditioned models for anxiety are relatively mild compared to the dramatic and consistent effects of 5-$HT_{1A}$ receptor activation and differences between inbred strains. Our data indicate that pharmacological studies with certain drug classes may be more feasible than previously thought. The generalization of this principle to other drug classes is an empirical question that needs to be addressed. A well balanced within-subject design using appropriate inter-test intervals significantly reduces the number of mice needed to complete studies employing dose–response curves. Giving serious ethical considerations towards animal health and welfare issues that require attention of the 3 R's of reduction, replacement and refinement, investigators should be encouraged to determine if a within-subject design can be utilized to address their research needs. Not only does the within-subject design result in a significant reduction in animal number requirements, but it also is more cost and space efficient and it increases the statistical power.

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