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The effect of three different items of cage furniture on the behaviour of male C57BL/6J mice in the plus-maze test

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Abstract

The aim of this study was to assess the effects of specific regimens of enrichment on the behaviour of C57BL/6J mice in the elevated plus-maze test (EPM). A total of 192 male C57BL/6J mice were allocated randomly to 32 cages. Three different items of cage furniture (CF) made of aspen — a mouse corner, nestbox and stairs — were added stepwise to different cages at intervals of one week so that the mice were exposed to an item of CF for one, two, three or four weeks. On the fifth week, all the mice were subjected to the EPM test. Overall, the presence of the nestbox or stairs for the three weeks appeared to have an anxiolytic effect on the behaviour of the mice, as evidenced by an increase in the number of entries made into the open arms and the time spent in the open arms of the EPM. The effects of these items of CF on the behaviour of the mice depended on the item used and on the duration of exposure. The items of CF that were used in this study appeared to improve the quality of life of C57BL/6J mice, as assessed using the EPM.

Keywords: *animal welfare, C57BL/6 mice, cage furniture, environmental enrichment, elevated plus-maze, housing refinement*

Introduction

Early definitions of environmental enrichment (EE) were made on the basis of a technical viewpoint, eg Hebb (1947) described EE as "any modification of a captive animal's environment by providing physical or social stimuli". Later definitions have emphasised the performance of the animal, hence they focus on the outcome of the procedure rather than the type of stimulus. For example, Belz *et al* (2003) defined EE as "using different objects to improve the quality of life of animals by distracting them from an otherwise monotonous environment", whereas Baumans (2005) defined it as "any modification in the environment of the captive animals that seeks to enhance physical and psychological well-being by providing stimuli meeting the animals' species-specific needs". Nonetheless, in considering the effects of items of cage furniture (CF) that are added to the environment of an animal, it is perhaps better to revert to the technical definition of EE, and to use the term 'cage furniture' (Olsson & Dahlborn 2002) because this term explains clearly the type of stimulus used.

The main aim of adding items to the environment of laboratory animals is to improve their quality of life. CF has numerous effects on the behaviour and physiology of rodents (for reviews, see Key & Hewett 2002; Olsson & Dahlborn 2002; Key 2004). For example, a decrease in the level of stress hormones (Belz *et al* 2003; Benaroya-Milshtein *et al* 2004), the attenuation of anxiety responses (Fox *et al* 2006), and a reduction in stereotypic behaviour (Turner *et al* 2003) have been reported.

CF should be considered an experimental variable (Hutchinson *et al* 2005) and may increase the variability of results (Van de Weerd *et al* 2002; Marashi *et al* 2004). Therefore, it may be difficult to compare results between studies with or without EE, or even between studies using different designs of CF (eg single versus many CF items). Indeed, it has been suggested that CF should be a component of a well-designed and critically evaluated programme that benefits the animals, in addition to having an effect on the outcome of the experiment (Baumans 2005).

The effect of CF on the behaviour of mice in the elevated plus-maze (EPM) test has been investigated in a number of studies. The results have ranged from an apparent anxiolytic effect (Caston *et al* 1999; Roy *et al* 2001; Benaroya-Milshtein *et al* 2004; Friske & Gammie 2005; Zhu *et al* 2006) to an apparent anxiogenic effect (Kobayashi *et al* 2006; Pietropaolo *et al* 2006), or no apparent effect

(Martinez-Cué *et al* 2002). These discrepancies can be ascribed at least partly to different combinations of CF items, different strains of mice, variable housing densities in the cage, and different cage sizes. These factors, as well as other unidentified parameters, complicate the comparison of the results of such studies. It is difficult to compare the effects caused by CF, because it is impossible to find studies that use the same CF items and cage size. For example, the area per mouse varies from 159 to 1,125 cm^2 in EE-housed mice (Friske & Gammie 2005; Kobayashi *et al* 2006). The ratio between the area of 'standard' housing (standard cages, without items of CF) and that of EE housing (larger cages or cage systems with different items of CF) also varies among different studies. To avoid these complications, we decided to use a very simple design and a single item of CF in each group.

Another factor, which is often neglected, is the cost that is associated with CF regimens. Nesting material, but not CF, is mandatory in Europe (Council of Europe 2006; European Union 2007). Intricate systems of CF exist that consist of numerous pipes and interconnected cages, which need to be dismantled and reassembled regularly. These systems are expensive to use, considering both the investment in materials and the labour that is associated with their maintenance. Simple autoclavable or disposable devices that can fit into the commonly used cages are more suitable for use as CF (Voipio *et al* 2008).

In our previous experiments, exposure of BALB/c mice to a Tapvei OY Mouse House (nestbox), (Kiili, Estonia) for 10 days did not alter the anxiolytic effect of 1-(2trifluoromethylphenyl)-imidazole (TRIM), a selective inhibitor of neuronal nitric oxide synthase (nNOS), in the EPM test. However, exposure for three weeks decreased the locomotor activity of mice in the EPM (Õkva *et al* 2007).

Since their introduction in 1913 and 1921, respectively (Wahlsten *et al* 2006), the BALB/c and C57BL strains of mice have been used extensively in research. According to the survey performed by Zhao *et al* (2007), C57BL and BALB/c mice were mentioned in 57,587 and 44,983 publications, respectively, from 1995 to 2005. Moreover, C57BL mice have been used as wild-type mice for the generation of genetically altered animals and F1 hybrids that may retain some characteristics of the parental strains (Kalueff *et al* 2007).

There are significant differences in behaviour between BALB/c and C57BL/6 mice: BALB/c mice display a higher level of anxiety (Kim *et al* 2002; Tang *et al* 2002; Ducotet & Belzung 2005) and lower sociability (for a review, see Brodkin 2007) than C57BL/6 mice. In the EPM test, the higher level of anxiety shown by BALB/c mice has been noted in both females (Augustsson *et al* 2005) and males (Lepicard *et al* 2000a; Augustsson & Meyerson 2004; Brooks *et al* 2005; Sunyer *et al* 2007). The BALB/c mice also exhibit elevated levels of corticosterone in response to stress (Priebe *et al* 2005), and they show limited exploration of a new environment when compared with C57BL/6J mice (Lepicard *et al* 2000a).

Various genetic and epigenetic factors (Francis *et al* 2003; Priebe *et al* 2005) have been suggested to account for the differences between BALB/c and C57BL/6 mice; for example, poor control of balance has been suggested to contribute to the anxiety-related behaviour of BALB/c mice (Lepicard *et al* 2000b).

Given that, in our earlier experiments, the presence of CF (a nestbox) had no major effect on the behaviour of BALB/c mice (Õkva *et al* 2007), we sought to discover whether this was also the case for C57BL/6 mice. Our aim was to compare the effects of different CF regimens on the behaviour of mice in a widely used model of exploratory activity — the EPM test. The main aim of this study was to assess the effect of three different items of CF, and four different periods of exposure, on behaviour in the EPM test.

Materials and methods

Ethics

The study protocol was reviewed and approved by the Committee that grants permits for the performance of animal experiments in the Republic of Estonia. The experimental procedures and the maintenance of the animals were in accordance with the Animal Welfare Act of the Republic of Estonia and the European Council Directive 86/609 EEC.

Study animals

One hundred and ninety-two naïve male C57BL/6J01aHsd mice (Harlan, Horst, The Netherlands) were used. The animals were three-weeks old on arrival, and were allowed to acclimatise for 18 days (ten days in quarantine and eight days in the animal room). The mice were ten-weeks old at the time of the EPM test, and weighed 24.6 (± 0.2) g. The mice were maintained at 21 (± 2)ºC and 50 (± 5)% relative humidity. No serious fighting in the cages or bite wounds on individual animals were observed. The mice were housed in a modern animal facility, which was assessed regularly according to the recommendations of the Federations of European Laboratory Animal Science Associations for health monitoring.

The mice were provided with pelleted food (Labfor R70, Lantmännen, Södertalje, Sweden) and autoclaved water, *ad libitum*. They were housed in groups of six in polycarbonate cages (Tecniplast, Buguggiate, Italy) that measured 42.5 × 26.6 × 15.0 cm (length × breadth × height) (Eurostandard type III), and were exposed to a 12:12h light/dark cycle, the lights were on from 0700 to 1900h. Autoclaved aspen chips (chip size 4 × 4 × 1 mm, Tapvei OY, Kiili, Estonia) were used as bedding (1 litre per cage).

Groups

The mice were allocated randomly to cages, with six animals per cage, to create the following groups. Each group consisted of two cages of mice. (i) Control housing — mice were group-housed in their home cages without any added items until the behavioural tests were performed. In order to control for any day-to-day variation in the experimental results, a separate control group was tested in parallel to the study groups on each day on which

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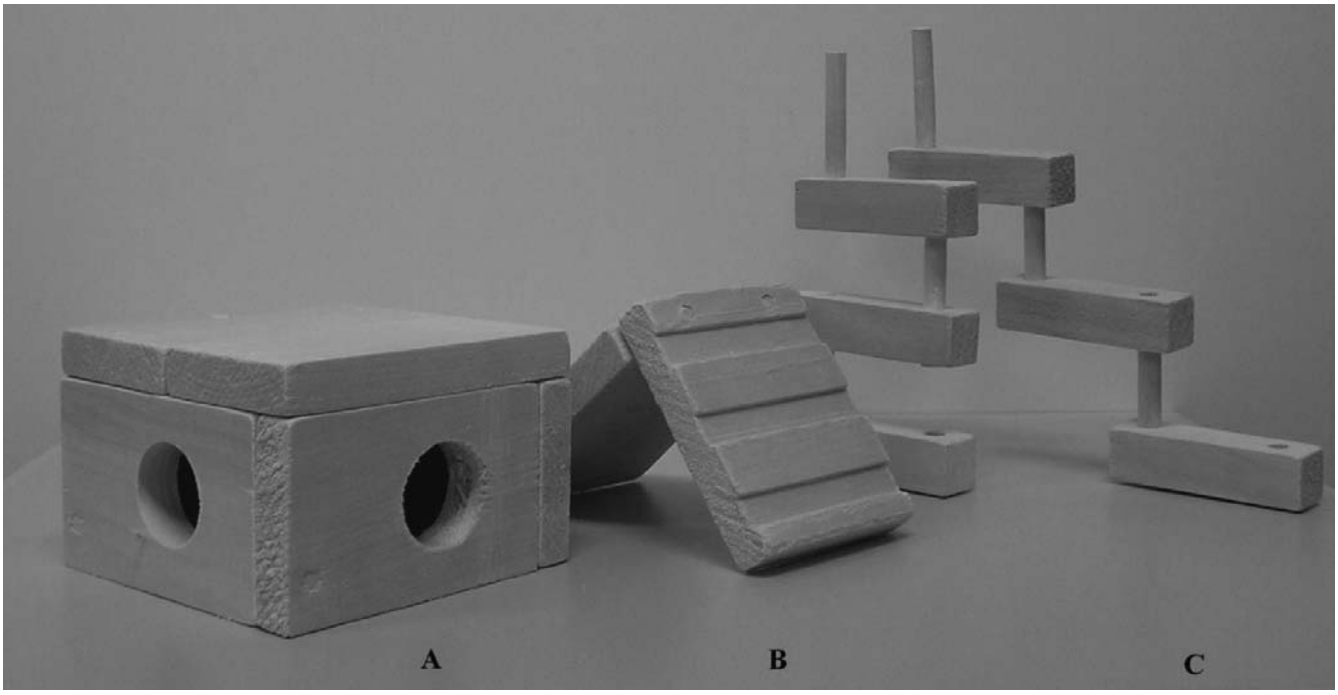
Table 1 Schematic illustration of the experimental protocol.

Group	1st week	2nd week	3rd week	4th week	5th week
Control	No CF	No CF	No CF	No CF	EPM
CF for 1 week				CF added	EPM
CF for 2 weeks			CF added		EPM
CF for 3 weeks		CF added			EPM
CF for 4 weeks	CF added				EPM

Figure 1



Items of cage furniture used in the study, (A) Tapvei OY nestbox, (B) Tapvei OY corner and (C) Tapvei OY stairs.

the EPM test was carried out. (ii) Added items — mice were group-housed in their home cages with one of three specific items of CF until the behavioural tests were performed. For each type of furniture, different groups were exposed to the item for one, two, three or four weeks (Table 1). The cages and the items were replaced by new cages and items of the same type once a week. The EPM test was performed on the four subsequent days. To reduce the risk of interactions, the mice in each group were divided randomly between different testing days and times.

The following items of CF were used: (i) Tapvei OY Mouse House (nestbox) — a quadrangular aspen box (110 × 110 × 70 mm), with walls 15-mm thick, and with two round openings (d = 30 mm) on two adjacent sides (Figure 1A); (ii) Tapvei OY Corner 15 (corner); this is formed from two aspen boards that are 15 mm thick (90 × 74 mm; length × breadth), and that are joined at an angle of 90°. On one of the outer surfaces of the structure there are three parallel indentations (2 mm deep, 12 mm wide; Figure 1B); and (iii) Tapvei OY Stairs (stairs) — these consisted originally of five rectangular aspen blocks (19 × 19 × 75 mm) that were connected by four aspen bars with a diameter of 7 mm and length of 70 mm. By removing two blocks from the original stairs, a stairs or a ladder was constructed, and two items — both the new stairs and the ladder — were used in a single cage (Figure 1C).

The plus-maze test

The animals were housed with other naïve mice in the same room. Animals were transported from their familiar animal room to the experimental room one hour before the EPM test and were allowed a period of habituation for approximately one hour. The tests were performed in two sets, each of which lasted for two hours, and which were started at

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Figure 2

Plus-maze apparatus. (the mouse in the picture was not involved in the current study).

1000 and 1400h, respectively. Prior to testing, the mice could not see the EPM apparatus. No other activities were permitted in the room. The EPM test was carried out in accordance with the methods described by Lister (1987).

The apparatus consisted of two open (8 × 17 cm) and two closed (8 × 17 × 30 cm) arms that were connected by a central platform (8 × 8 cm) that was elevated 30 cm above the ground (Figure 2). The mice were placed on the central platform, facing an open arm. The behavioural parameters were recorded by an experienced person in the room. The number of entries that were made into the open and the closed arms during a period of five minutes was counted. From these data, the percentage of entries that were made into the open arms, and the percentage of time that was spent in the open arms, was calculated. The EPM was cleaned thoroughly, before the introduction of each animal, with an antiseptic solution of 1% Virkon®S (Antec™ International, Suffolk, UK).

Statistical analysis

The data were analysed using univariate analysis of variance (ANOVA) with SPSS 14.0 software, and the Tukey's test was used for the *post hoc* comparisons. Comparisons were made among 13 groups, ie the control group and the groups that had been exposed to one of the three items of CF (corner, nestbox or stairs) for four different exposure times (one, two, three or four weeks). Levene's test was used to assess whether there were differences in variance among the groups.

Results

In the control group, the number of entries that were made into the open arms was 2.7 (± 0.3); the total number of entries into the closed and the open arms was 13.0 (± 0.8). In this group, the percentage of entries that were made into the open arms of the EPM was 21.2 (± 2.6)% and the percentage of time that was spent in the open arms of the EPM was 4.6 (± 0.6)% (see Figure 3).

The data on entries into the open arms and the time that was spent in the open arms did not follow a normal distribution, but the residuals from the ANOVA did follow a normal distribution. As a consequence, statistical analysis of the two original parameters was performed with logarithmically transformed values, but the results are

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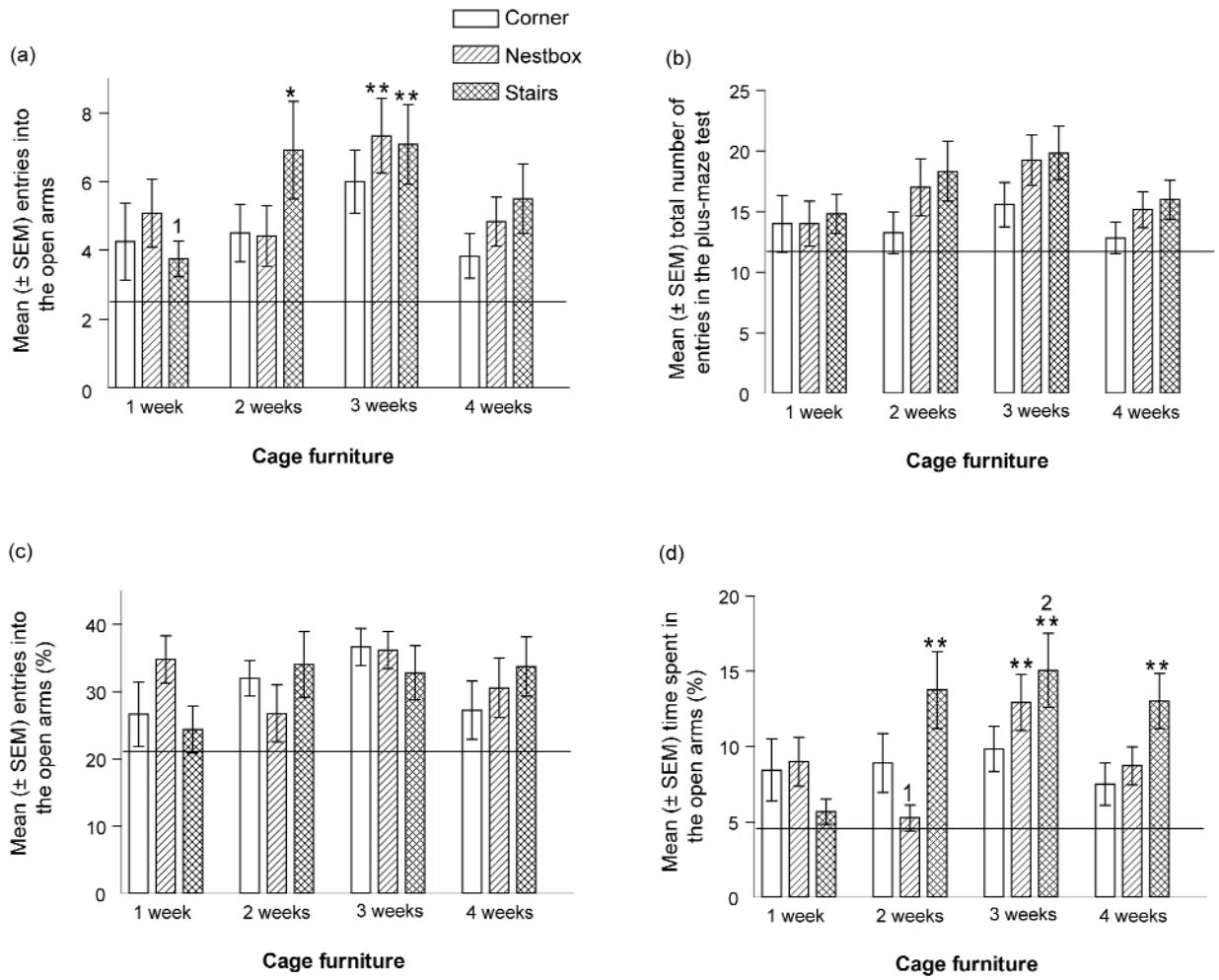
Figure 3



The effect of specific items of CF on the behaviour of mice in the EPM test. Data are presented as the mean (± SEM) from groups of 12 mice. This figure shows (a) the number of entries made into the open arms, (b) the total number of entries made in the EPM test, (c) the percentage of entries made into the open arms and (d) the percentage of time spent in the open arms. Lines across the bars show representative means for the control (no item) group (n = 48). * $P < 0.05$ vs control, ** $P < 0.01$ vs control (contrast analysis). 1 — Significantly different as compared with the stairs for 2 weeks and 4 weeks. 2 — Significantly different as compared with the stairs for 1 week.

given as means (± SEM). The effect of CF depended not only on the type of CF used but also on the length of exposure (Figure 3).

Mice that had been exposed in their cage to the stairs for two or three weeks (but not for one or four weeks) made significantly more entries into the open arms than the control mice. Exposure to stairs for two, three or four weeks (but not for one week) resulted in a significantly higher percentage of time spent in the open arms, when compared with the controls.

Exposure to the nestbox for three weeks (but not for one, two or four weeks) resulted in significantly more entries made into the open arms and also in a significantly higher percentage of time spent in the open arms as compared with the control mice.

The presence of the corner in the cage for one, two, three or four weeks was associated with no statistically significant changes. None of the items of CF, when provided for any of the exposure times, was associated with any significant changes in the total number of entries into the closed and open arms or in the percentage of entries into the open arms.

Finally, provision of either the stairs or the nestbox for three weeks (but not for one, two or four weeks) caused a significant decrease of weight gain (Figure 4).

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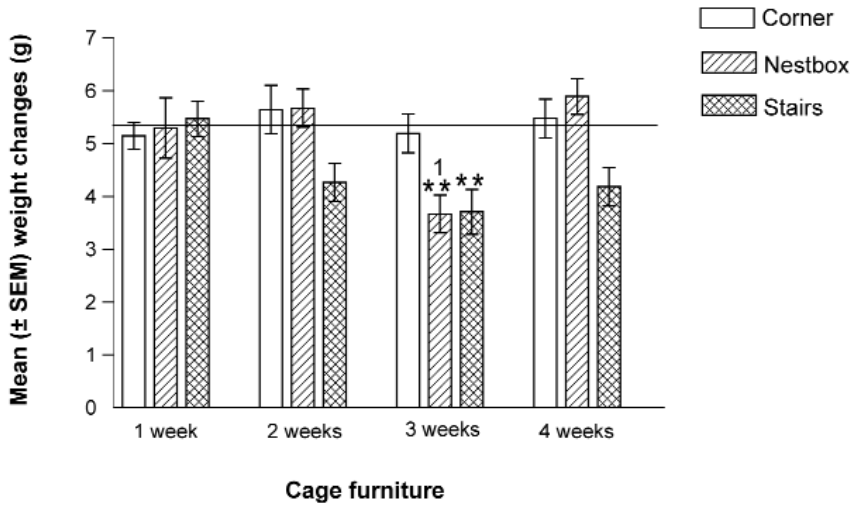
Figure 4



The effect of specific items of CF on weight change during a four-week period. Data are presented as the mean (± SEM) from groups of 12 mice. The line across the bars represents the mean for the controls (no furniture). ** $P < 0.01$ vs control (contrast analysis), (n = 48). 1 — Significantly different as compared with the nestbox for 2 weeks ($P < 0.05$) and 4 weeks ($P < 0.01$).

Discussion

The study described herein shows that the exposure of mice to the types of CF that were used increased the number of entries into the open arms of the EPM test; it also increased the percentage of time spent in these arms. Exposure to CF also tended to increase the total number of entries. This effect seemed to be limited in time; it increased until the third week and decreased again on the fourth week. The apparent anxiolytic effect was most pronounced in the third week; two of the three items of CF had a statistically significant effect on the number of entries that were made into the open arms of the maze, and on the percentage of time that was spent in the open arms, after an exposure period of three weeks.

Since the EPM test was validated in rats (Pellow *et al* 1985) and in mice (Lister 1987), it has been shown repeatedly that anxiolytic drugs increase the percentage of entries that are made into and the percentage of time that is spent in the open arms of the EPM. Anxiogenic drugs have the opposite effect. Therefore, it appears that the addition of CF items to the cage induces an anxiolytic effect in the EPM test. This is likely to enhance animal welfare, because stress has been associated with the appearance of anxiety in the EPM test (Hsu *et al* 2007; Sterlemann *et al* 2008).

The results that have been obtained in studies of the effect of CF on mouse behaviour in the EPM test have been somewhat conflicting. All outcomes seem to be possible; CF may result in no effect (Martinez-Cué *et al* 2005), an apparent anxiolytic effect (Caston *et al* 1999; Roy *et al* 2001; Benaroya-Milshtein *et al* 2004; Friske & Gammie 2005; Zhu *et al* 2006), or an apparent anxiogenic effect (Kobayashi *et al* 2006; Pietropaolo *et al* 2006). The different behavioural profiles observed may be due to the large variety of types of CF used in these studies. However, this may be too simplistic, because these discrepancies may also be attributable to the use of different strains of mice, the sex of the animals, or other as yet unidentified factors. Indeed, it has been shown that the effects of CF on the behaviour of animals depend on the age (Harburger *et al* 2007; Mirochnic *et al* 2009) and sex (Elliott & Grunberg 2005; Pena *et al* 2006) of the animals, and on the line of outbred mice (Fernandez-Teruel *et al* 2002) or the strain of inbred mice (Tucci *et al* 2006) that is used in the experiments. We performed the EPM test when the animals were the same age (10 weeks old). Hence, the age of the mice when they were first exposed to CF varied among the groups that were exposed to CF for different times. We were unable to find any studies on how the effects of age and EE on the behaviour of mice interact. There are reports that discuss age, EE, and neurodegeneration or neurogenesis. However, in these articles, the age differences were significantly greater than in our study, for example in the study of Harburger *et al* (2007) the mice were 3, 15 or 21 months old. In summary, we cannot rule out that the apparent time-course of changes observed in the current study was not caused by the period of exposure alone, but also by the different ages of the mice at the time of their first exposure to CF.

To complicate the picture further, there is evidence that opposite effects may be seen with different types of CF. For example, in a Dutch study, the provision of nesting material reduced, whereas shelter increased, aggressive behaviour in BALB/c mice (Van Loo *et al* 2002). Moreover, different items of CF have been associated with a variety of effects on spatial memory (Bennett *et al* 2006). It is noteworthy that, in the majority of reports that deal with the effects of CF on mouse behaviour, the effect of the duration of exposure to the CF has not been examined. Therefore, it cannot be excluded that these apparently contradictory results are related to the specific time-course of the changes.

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It is difficult to explain the discrepancies between different studies because it is extremely difficult to find two studies that use identical or even similar regimens of CF. For instance, surprisingly large differences in housing densities (ranging from two- to eight-fold) are common; mice that are exposed to CF usually have much more floor space than those without items of CF (Wolfer *et al* 2004). Furthermore, in the majority of studies, the description of the CF used is vague, which makes exact reproduction impossible.

In our previous studies, the provision of CF, in the form of a Tapvei Mouse House (nestbox), had no effect after 10 days of exposure, but it reduced the locomotor activity of BALB/c mice significantly after 21 days. The provision of a nestbox, irrespective of the duration of exposure, did not induce an apparent anxiolytic effect in the EPM test (Õkva *et al* 2007). It has been demonstrated repeatedly that C57BL/6 mice are less anxious than BALB/c mice in the EPM test (Augustsson & Meyerson 2004; Brooks *et al* 2005). It may be that the apparent anxiolytic effect of the CF was not sufficiently strong to overcome the high level of anxiety that is characteristic of BALB/c mice.

The effect of CF depends not only on the type of object that is used but also on the length of exposure. This finding is relevant to the design of the housing environment for behavioural studies. The typical period of quarantine in animal facilities is 10 to 14 days. Therefore, scientists should take into account the duration of exposure and the type of item(s) to which the animals are exposed and also that the effect of CF might be limited by time. It is possible that the effect of CF is based on novelty and therefore diminishes gradually. This hypothesis is supported by the findings of de Visser *et al* (2005), who showed that activity on a running wheel decreased at a constant rate over six days.

In this study, some of the CF that was used was associated with a temporary decrease in the rate of gain of bodyweight. Again, contradictory data can be found on the effects of CF on bodyweight gain; studies have reported increased (Van de Weerd *et al* 2002; Augustsson *et al* 2003; Meijer *et al* 2007), reduced (Haemisch & Gärtner 1994; Kaliste *et al* 2006), or unchanged (Tsai *et al* 2002; Van der Meer *et al* 2004) weight gain. The possible significance of this reduced weight gain to animal welfare is unclear. One possible explanation of this effect is that CF distracts the animals from the otherwise monotonous environment.

Conclusion

The items of CF that were used in this study seemed to result in a refinement of the housing systems that are used for C57BL/6J mice, as assessed by the EPM test. The effect of CF on the behaviour of mice in the EPM test depends on the type of objects and is limited by time.

Acknowledgements

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