Prolonged Exposure of Mice to a Nest Box Reduces Locomotor Activity in the Plus-Maze Test

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Summary

Environmental enrichment (EE) has been associated with many effects on the behavior of laboratory animals. The term EE is rather vague, often referring to a variety of item combinations as if what is added to the cage has no significance. EE is indeed housing refinement, and therefore more exact terms should be used to clarify the situation. This study was designed to assess whether access to a nest box (NB) could modify behavior of BALB/c mice in the plus-maze test. Two series of experiments were done with an aspen NB (11 x 11 x 7 cm, wall thickness 1.5 cm, two round holes (d = 3 cm) at opposite sides. Control mice had no added item in the cage. The plus-maze consisted of two open (8 x 17 cm) and two closed arms (8 x 17 x 30 cm) connected by a central platform (8 x 8 cm). Mice were placed on the central platform facing an open arm. During five minutes, the numbers of entries made onto the open and into the closed arms were recorded. From this data, the percentages of entries made onto the open arms, and the percentage of time spent on the open arms, were calculated. Furthermore, the number of fecal boli left by the mice in the plus-maze, as a stress indicator, were counted. In the first series of experiments NB was present for one, two and three weeks but no drugs were administered. NB provided for one or two weeks had no effect on the behavior of mice. However, exposure to NB for three weeks did decrease the locomotor activity of mice in the plus-maze test, as reflected in the decline in the total number of entries made in the test. The presence of NB for one or two weeks resulted in more ($p = 0.001$) fecal boli voided when compared to the no NB or NB for three weeks groups.

In the second series of experiments we used NB for 10 days and the selective neuronal nitric oxide synthase (nNOS) inhibitor 1-(2-trifluoromethylphenyl)-imidazole (TRIM) as a pharmacological tool (at doses of 25.0, 50.0 and 100.0 mg/kg, i.p.). Depending on the dose, the administration of TRIM induced an anxiolytic (50 mg/kg) or sedative effect (100 mg/kg) as seen in the increase in the percentage of entries made onto the open arms or a decrease in the total number of entries, respectively. NB for 10 days had no effect on the behavior of mice or on the effect of TRIM. In conclusion, NB does not appear to interfere with the anxiolytic effect of TRIM in the plus-maze test but prolonged exposure to NB does reduce the locomotor activity of mice.

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Introduction

The Council of Europe Appendix A sets the minimum requirements for environmental complexity

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available to laboratory animals (*Council of Europe 2006*). They state that rodents should be provided with enough nesting material to build a complete, covered nest. If this cannot be fulfilled, then the animals should be given a nest box (NB). The mouse as a species is a good nest builder, more so than rats.

Surprisingly, little has apparently been published on the effects of NB alone on mouse welfare and behavior. In most studies, a NB has been used in conjunction with various other items such as tissue, wood wool, PVC tube and gnawing block (*Augustsson et al., 2003*), wheel (*Chapillon et al., 1999*), cotton nestlets (*Coviello-McLaughlin et al., 1997*), paper strips (*Emond et al., 2002*), wooden balls and slats (*Friske & Gammie, 2005*), tissue (*Heizmann et al., 1998*) marble and split pipe (*Hobbs et al., 1997*), nesting material (*Kaliste et al., 2006*), tubes and a running wheel, toys (*Kobayashi et al., 2006*), paper, wooden blocks and platform (*Leach et al., 2000*), plastic and wooden tunnels and plastic toys (*Pietropaolo et al., 2006*), tissues, wood wool, grid floor and tube *(Van de Weerd et al., 1994*), tissue, blocks, tube and wood wool (*Van de Weerd et al., 2002*), cotton nestlets and wooden bar (*Tsai et al., 2002; Tsai et al., 2003a,b*), wooden tunnels and running wheels (*Zhu et al., 2006*). It is possible that all these items may have contributed to the results.

Environmental enrichment (EE) has been associated with numerous effects on the behavior of laboratory animals (for reviews see *Key & Hewett, 2002; Key, 2004; Olsson & Dahlborn, 2002*). Nesting material and NBs are often dealt under the heading of EE. This term is quite vague, and if it is defined as performance, as is the case in more recently published articles, then it is hard to see how it can be used as an exposure. Furthermore, EE is most commonly a variety of item combinations as if the actual items placed into the cage would make little difference to the overall results. EE is indeed housing refinement, and therefore more exact terms should be used to indicate their individual impact.

All housing refinements should be beneficial to the animals, and if they are not, they are worthless. Neither should the impact of housing refinement on the studies themselves be negative, because this may interfere with the interpretation of the results. Furthermore, we should not simply accept the 'no effect' scenario in science, but rather search for those refinements in housing which contribute to better science. By combining the effects on welfare and science we should strive to find ways to rank housing refinements.

Baumans (*2005*) urged scientists to compile, document and publish pertinent data to dispel the myths and define the variations related to EE. There is a requirement for well-designed and carefully communicated housing refinement approaches, but those have to focus on a limited number of commonly used items and use a similar methodology. If this is not achieved, then integration of the results, including meta-analysis, will not be possible. In other words, it should not be a process of randomly applying objects that the staff considers might be attractive to the animals (*Baumans 2005*).

Nitric oxide (NO) was the first of an entire family of unusual neurotransmitter molecules (*Dawson & Dawson, 1994*), synthesized on demand by the enzyme NO synthase (NOS) and since it is gas, it diffuses out of nerve terminals (*Esplugues, 2002*). Three separate NOS genes and the corresponding enzymes have been identified and named either by the tissue or the order in which they were cloned (*Yun et al., 1996*) – neuronal NOS (nNOS, Type I NOS), immunological NOS (iNOS, Type II NOS) and endothelial NOS (eNOS, Type III NOS). nNOS has many functions in the central and peripheral nervous system (for review see *Esplugues, 2002*). For example, NO is also involved in the regulation of anxiety (for review see *Guimarães et al., 2005*) and sleep (*Monti et al., 1999*).

Since the presence of a NB could have some effect on behavioral studies it was of interest to study its impact and to find out whether the NB could modify the effects of a selective neuronal nitric oxide synthase (nNOS) inhibitor 1-(2-trifluoromethylphenyl)-imidazole (TRIM) in the elevated

plus-maze test. This study was designed to assess the effect of NB either alone or combined with a selective nNOS inhibitor TRIM in this widely used model of exploratory behavior.

Materials and Methods

Ethics

This study protocol was reviewed and approved by the Committee granting permits for conducting animal experiments in the Republic of Estonia.

Animals

Naive male inbred BALB/c/Bkl (Scanbur BK, Sollentuna, Sweden) mice weighing 23.0 ± 1.2 g (mean ± SEM) were used. They were maintained at 21 ± 2 ºC and 50 ± 5 % relative humidity with autoclaved water and food (Labfor R70, Lactamin, Södertalje, Sweden) available *ad libitum* and housed in groups of ten in polycarbonate cages (Tecniplast, Buguggiate, Italy) measuring 42.5 x 26.6 x 15.0 cm (Eurostandard type III) and exposed to a 12/12 h light/dark cycle. Lights were on from 07:00 to 19:00. Autoclaved aspen chips (chip size 4 x 4 x 1 mm, TapveiOY, Kiili, Estonia) were used as bedding.

Groups

The study was carried out as two series of experiment. In the first series, NB was provided for one, two and three weeks without any pharmacological intervention; the second series used NB for ten days combined with a selective nNOS inhibitor TRIM as a pharmacological tool.

Series 1. Time course of effects of NB

The following groups of mice were used (Table 1):
(1) Control housing – mice group-housed in cages until the behavioral tests.
(2) Nest box – mice group-housed in cages with NB for one, two or three weeks until the behavioral test.

Series 2. Effect of TRIM and NB on the behavior of the mice

The following groups, both with vehicle treatment and three doses of TRIM (25, 50, 100 mg/kg), of mice were used:
(1) Control housing – mice group-housed in cages until the behavioral tests.
(2) Nest box – mice group-housed in cages with NB until the behavioral test.
A Tapvei OY mouse house was used as the NB; it is a quadrangular aspen box with external measures 11 x 11 x 7 cm (l x w x h), 1.5 cm thick walls and two round holes (d = 3 cm) at opposite sides. From both groups of series 2, mice were assigned to either the vehicle or TRIM treatments. Once the injections were completed, the mice were returned to their cages.

Group	1st week	2nd week	3rd week	4th week
Control housing	No addition			Plus- maze
NB for one week			NB added	Plus- maze
NB for two weeks		NB added		Plus- maze
NB for three weeks	NB added			Plus- maze

Table 1. Study design of Series 1. There were ten animals in each of the four groups.
Abbreviation: NB = nest box.

Pharmaceuticals

TRIM (Sigma, St. Louis, MO, USA) was dissolved in saline. TRIM or saline were administered intraperitoneally at a volume of 0.1 ml / 10 g of body weight, 60 minutes before the test.

The plus-maze test

Animals were transported from their familiar animal room to the experimental room one hour before the plus-maze test in order to allow habituation. The mice could not see the plus-maze apparatus. The plus-maze test was carried out with only a minimal amount of background noise from the ventilation system. No other activities were going on in the room. The plus-maze test was carried out according to Lister (*1987*). The plus-maze consisted of two open (8.0 x 17.0 cm) and two closed arms (8.0 x 17.0 x 30.0 cm), which were connected by a central platform (8.0 x 8.0 cm). The plus-maze was elevated 30 cm above the floor level. Mice were placed on the central platform facing an open arm. After each test the plus-maze was thoroughly cleaned with antiseptic solution (1% Virkon®S (Antec™ International, Suffolk, England). During five minutes, the numbers of entries made onto the open and into the closed arms were recorded. From the resulting data, the percentages of entries made onto the open arms, and the percentage of time spent on the open arms, were calculated.

Data analysis

All data was tested for normal distribution with the Kolmogorov-Smirnov test. The data, which differed from normal distribution, were subjected to logarithmic transformation, and the normality of the resulting data was assured. Subsequently, all behavioral data of animals in the plus-maze test was analyzed using One-Way (series 1) or Two-Way (series 2) Analysis of Variance (ANOVA) using TRIM and NB as factors. Further statistical analysis was conducted using contrast analysis.

Absolute deviations of all values from respective means of all parameters were tested with Levene's test of equality of error variances. Statistical significance was set at $p = 0.05$.

Results

Series 1.

NB for one, two or three weeks had a significant effect on the number of entries made into the closed arms [$F(3.36) = 3.24$, $P < 0.05$] and showed an almost statistically significant trend on the total number of entries made in the plus-maze test [$F(3.36) = 2.76$, $P < 0.056$]. Further analysis revealed that the NB for three weeks significantly ($p = 0.02$) decreased the number of entries made into the closed arms as compared to the control housing group (Figure 1).

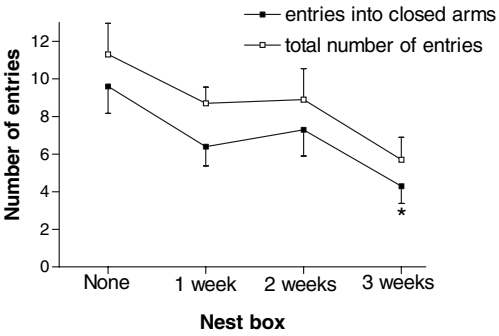

Figure 1. The time-course effect of nest box on the behavior of mice. Data are presented as mean ± SEM from groups of ten mice. Abbreviations: * = $P < 0.05$ vs. the "None" (i.e. control) group (contrast analysis).

Levene's test indicated that standard deviation (SD) of time spent in open arms was significantly less with NB for two weeks in the cage as compared to all other groups, but when this was processed to obtain coefficient of variation, then the values from all the groups were almost identical.

The numbers of fecal boli (mean ± SEM) were 3.20 ± 0.74, 6.50 ± 0.73, 6.20 ± 0.81 and 3.30 ± 0.52 in the control housing group and NB for 1, 2 and 3 weeks groups, respectively. The presence of NB for one or two weeks resulted in more ($p = 0.001$) fecal boli voided when compared to the control housing or the NB for three weeks groups.

Series 2.

Two-way ANOVA demonstrated a significant effect of TRIM on the number of entries made onto the open arms [F (3.39) = 5.97, p < 0.001], into the closed arms [F (3.39) = 7.66, p < 0.001)] and on the total number of entries made in the plus-maze test [F (3.39) = 8.52, p < 0.001]. NB for ten days had no effect on the behavior of mice; but these results suffer from poor statistical power (p<0.09). Further contrast analysis revealed that TRIM, administered 60 min before the experiment, had a dose-dependent effect on the behavior of mice in the plus-maze test (Figure 2).

The low dose of TRIM (25 mg/kg) had no effect on anxiety levels in the plus-maze test. The medium dose (50 mg/kg) induced an anxiolytic effect as evidenced by a significant (p = 0.004) increase in the number of entries made onto the open arms and in the percentage of entries made onto the open arms of the plus-maze. The high dose (100 mg/kg) evoked a sedative effect as verified by a significant (p = 0.01) decrease in the number of entries made into the closed arms and in the total number of entries (Figure 2). The effect of TRIM was similar in control housing and NB groups.

Discussion

There is a definite need to evaluate the various housing refinements for animals used for scientific purposes, commonly called environmental enrich-

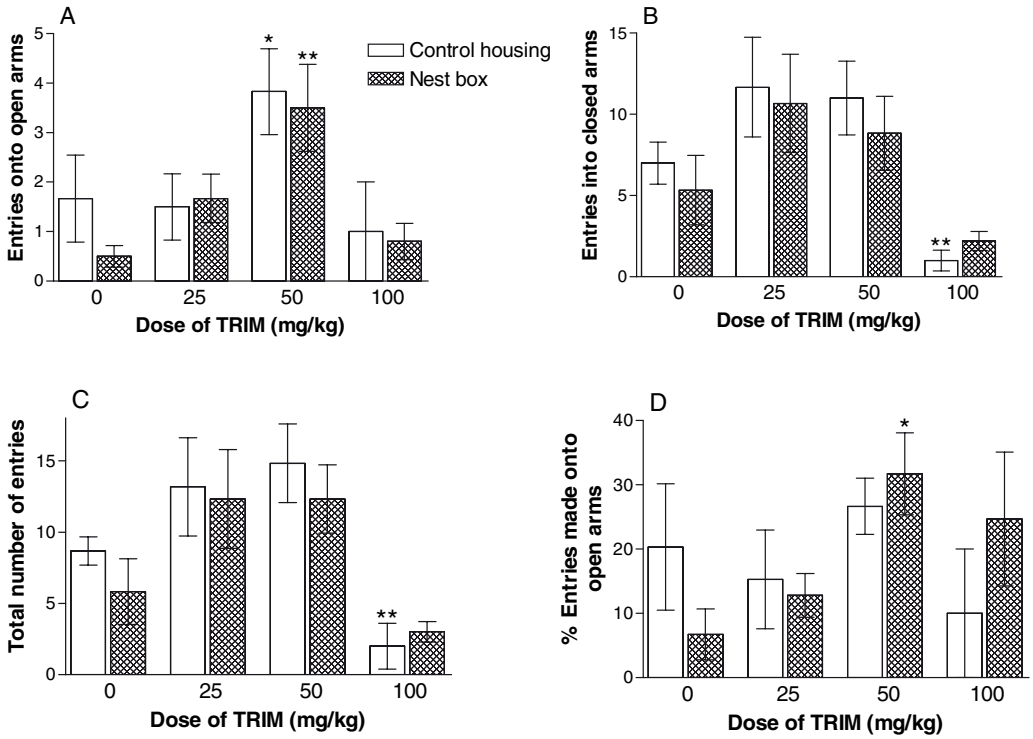

Figure 2. The effect of TRIM on the behaviour of control housing and nest box on mice in the plus-maze test. Data are presented as mean ± SEM from groups of six mice. This figure shows the number of entries made onto the open arms (A), the number of entries made into closed arms (B), the total number of entries (C) and the percentage of entries made onto the open arms (D). Abbreviations: * P < 0.05 vs. vehicle, ** P < 0.01 vs. vehicle (contrast analysis)

ment (EE). Ideally these improvements should be evidence-based and allow ranking of items intended or used for the purpose. In contrast to practice in other disciplines, *e.g.* in pharmacology, it is rather common to assess the effects of item combinations which makes it impossible to evaluate the effect of a single object.

In addition to animal welfare, it is necessary to safeguard the integrity and validity of research being conducted with the animals. EE has been associated with opportunities to enjoy normal species-specific behavior, but it has also been claimed that animals exposed to new items in the cage behave differently from before (*Smith, 2005*). The latter - if true - may render much of the previous data from behavioral studies irrelevant.

Another potential interference may arise from significant changes in result variation. A change in result means may be indeed be the lesser of the evils, since it is balanced by simultaneous controls enjoying the same housing. In this study we observed one group in series 1 with significantly lower variation in open arms entries. If this absolute difference - as is the usual approach - were to be processed with power analysis to calculate the appropriate numbers of animals needed in that group, much fewer animals would be needed, even though the relative variations were essentially the same in all the groups.

Baumans (*2005*) emphasized the requirement for well-designed and carefully communicated housing refinement approaches. Therefore, EE should focus on a limited number of commonly used items and be based on a similar methodology. This study focused on NB only; hence the results may serve as a contribution to understanding the effect of NB, used either alone or in combination with other items.

The aspen NB was chosen for several reasons. Using a box made of the same material as bedding – aspen wood – eliminates problems that may arise when introducing new materials in the cage, to which the animals will be chronically exposed. Materials made of recycled paper mass may contain toners and other deleterious residues. Even items made of used polycarbonate equipment may evoke estrogenic effects (*Howdeshell et al., 2003*). Furthermore, since mice shred nesting material, it is possible that this may produce a larger amount of dust, which may interfere with the IVC system operation. Last, but not least, NB also provides a place for the mice to hide.

Wooden NBs may be considered as being difficult to clean, and hence regarded as disposable. This, however, is not necessarily true, they can be sanitized and reused several times; and they even endure several cycles of autoclaving (*Voipio et al.,* manuscript). One disadvantage is that mice are harder to observe inside boxes with non-transparent walls.

This study chose to use BALB/c mice because it has been shown that they exhibit a high level of anxiety in the plus-maze test (*Brooks et al., 2005*). When animals were exposed to the NB for 21 days, the locomotor activity of mice decreased. This study did not detect changes in the majority of plus-maze indices. The only difference was in the number of fecal boli left in the plus-maze. Mice exposed to the NB for one or two weeks left significantly more fecal boli when compared to no NB or NB box for three weeks groups. It has been proposed that increased defecation reflects the stimulation of autonomic responses to stress (*Swiergiel & Dunn 2006*) and that acute stress increases the number of fecal boli left in the plus-maze apparatus by mice (*Calvo-Torrent et al., 1999*).

The results published on NB effects on anxiety are rather diverse. Friske and Gammie (*2005*) and Zhu *et al.,* (*2006*) claim to have observed an anxiolytic effect, whereas Kobayashi *et al.,* (*2006*) and Pietropaolo *et al.,* (*2006*) conclude that the opposite, an anxiogenic effect, is true in the plus-maze test. However, in those studies, the NB was used in combination with various other items, such as tubes or tunnels (*Kobayashi et al., 2006; Pietropaolo et al., 2006; Zhu et al., 2006*), running wheels (*Kobayashi et al., 2006; Zhu et al., 2006*), toys (*Kobayashi et al., 2006; Pietropaolo et al., 2006*)

etc. In addition, the mouse strains and sexes vary in the different studies. Any of those many factors, alone or in combination, may explain discrepancies reported between this present and the published studies.

In this study NB when available for ten days had no effect on the behavior of mice in the plus-maze test, *i.e.* the indices of exploratory activity – the number of entries onto open and into closed arms and time spent on the open arms – were unchanged in mice exposed to the NB.

The selective nNOS inhibitor TRIM induced a dose-dependent anxiolytic effect in the plus-maze test as reflected in the increase in the number and percentage of entries made onto the open arms. Since the validation of the plus-maze test in rats (*Pellow et al., 1985*) and mice (*Lister 1987*), it has been repeatedly shown that anxiolytic drugs increase the percentage of entries made onto and the percentage of time spent on the open arms of the plus-maze and conversely that anxiogenic drugs decrease these measures.

These data are in line with previous study results demonstrating the anxiolytic effect of TRIM in another model of exploratory behavior – the light-dark compartment test (*Volke et al., 2003*). This study also confirms the participation of nNOS subtype in the regulation of anxiety.

It is worth mentioning that NB had no influence on the effects of TRIM in the plus-maze test, although the associated statistical power was poor. This suggests that NB does not interfere with the anxiolytic effect of TRIM and can be applied as EE in mice being used in the plus-maze test. On the basis of our data, it can also be concluded that the effect of NB seems to depend on the exposure time.

It should be also stressed that used EE should be clearly and explicitly described in order to enable the exact reproduction of experimental setup and comparison of results. Terms too often found in scientific articles, like assorted items and lists ending with *etc*., are simply not acceptable. It is obvious that more studies are needed and these should include comparisons with ranking of different EE items. If this is not done, integration of EE results will remain a difficult task.

The effect of NB depended on the length of exposure, as evidenced by the lack of effect after 10 days, but reduced locomotor activity after prolonged exposure. We conclude that a selective nNOS inhibitor, TRIM, induces an anxiolytic effect in the plus-maze test, thus confirming the participation of the nNOS subtype in the regulation of anxiety, but the anxiolytic effect of TRIM is not influenced by NB exposure.

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