An environmental enrichment model for mice

Yehezkel Sztainberg[1,2] & Alon Chen[1]

[1]Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel. [2]Gonda Brain Research Center, Bar-Ilan University, Ramat-Gan, Israel. Correspondence should be addressed to A.C. (alon.chen@weizmann.ac.il).

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Environmental enrichment for animals is a combination of complex inanimate and social stimulation and generally consists of housing conditions that facilitate enhanced sensory, cognitive, motor and social stimulation relative to standard housing conditions. One of the most robust effects of environmental enrichment is the reduction of anxiety levels. However, the extreme variability in enrichment protocols may account for some of the inconsistencies in its effects and the variance among results reported by different laboratories. In this protocol, we describe a simple environmental enrichment strategy for the induction of a robust and replicable anxiolytic-like effect in mice. We provide detailed instructions on how to build an enrichment cage that is specially designed for easy manipulation, cleaning and observation by the experimenter. In addition, we describe the different enrichment items, their order in the cage, the frequency of renewal and their cleaning and sterilization procedures. The total length of the protocol is 6 weeks.

INTRODUCTION

Environmental enrichment (EE), classically defined as 'a combination of complex inanimate and social stimulation'[1], typically consists of housing animals in large groups in relatively spacious and complex cages, with a variety of objects (e.g., nesting material, running wheels and tunnels) that facilitate enhanced sensory, cognitive, motor and social stimulation relative to standard laboratory housing conditions. Most important, an enriched environment provides the animals with opportunities to perform some of their species-specific behavioral repertoire. Since the pioneering experiments by Rosenzweig and colleagues[2,3], who introduced EE as an experimental protocol, many studies have demonstrated that environmental stimulation elicits various positive effects on the brain at the molecular, anatomical and behavioral levels (for reviews, see refs. 4–6), such as an increase in hippocampal neurogenesis[4,7], enhanced learning and memory[4,8,9] and the induction of neural plasticity[10,11].

The design and composition of the EE protocol vary widely between laboratories and are often not fully described. This variance may account for some of the inconsistencies among results reported by different studies[12]. Different stimulus objects are used, such as shelters and toys, and they usually vary in shape, color, texture and size. In each study, the selection of enrichment items may depend on the type of experience the experimenter wants to provide to the animals, according to the scientific questions of the study (e.g., social, cognitive, motor, sensory). The degree of complexity may also vary between studies, from the simple addition of one or two items to a standard cage to the use of specially designed complex enrichment cages containing a variety of objects. Other sources of variation across studies are species, sex, age and the number of animals housed together. The procedure of the EE protocol, which includes the duration of enrichment, the frequency with which the objects and their position are changed and whether the animals are exposed 24 h per day to EE or restricted to only a few minutes to a few hours a day, constitutes yet another potential confound. As a successful EE procedure design depends on the species, sex and age under investigation, it is difficult to standardize EE protocols. However, a basic EE protocol that can be easily adapted for use in a wide range of applications may provide investigators with a good starting point for EE studies. The protocol presented here has been used by our laboratory for the past 3 years as a simple and nonpharmacological method to induce a state of reduced anxiety[13] and reveal molecular targets for the development of therapeutic agents that mimic or enhance the beneficial effects of enrichment on stress-induced behavioral and neuroendocrine changes[14].

Components of EE

It has been suggested that physical exercise is a critical component of enrichment, and this hypothesis is supported by the fact that in some aspects exercise and enrichment are similar in their behavioral and morphological effects[4]. For example, both can increase adult hippocampal neurogenesis and improve spatial learning ability[7,15]. The effect of EE and exercise on neurogenesis appear to be synergistic, as exercise increases the level of proliferation of progenitor cells, whereas EE affects cell survival[16,17]. Another important component of EE is social interaction. Rodents are highly social, and social contact with conspecifics is their most challenging enrichment factor. With social partners, in contrast to static enrichment objects, animals can perform social behaviors such as mutual grooming, social exploration, vocalizations and play[18]. The provision of nesting material is considered to be another fundamental component of the EE design. Nesting material is highly valued by mice[19] and both males and females will build a nest when offered nesting materials[20]. Nests are important for heat conservation and reproduction; they also enable the animals to shape their own microenvironment to provide shelter[21].

In this protocol, we have adopted a complex enrichment strategy in which several enrichment items are combined to cover a broad span of enrichment effects. The emphasis was placed on the development of an EE protocol that provides animals with opportunities to perform their species-specific behaviors so as to induce a robust anxiolytic-like effect.

Experimental design

In our laboratory, a special EE cage was designed for easy manipulation, cleaning and observation by the experimenter. The EE cage (**Figs. 1** and **2**) consists of a transparent plastic box of 86 × 76 × 24.1 cm. The lid of the cage was specially designed to hold a standard food and

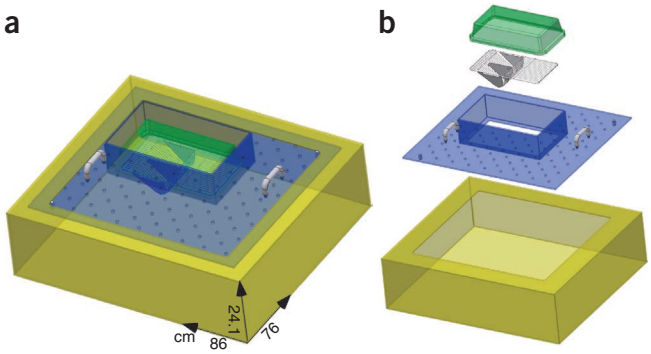

Figure 1 | EE cage design. (**a**) Perspective view of the EE cage. The colors of the cage are for illustration purposes only. (**b**) Exploded view of the EE cage, highlighting its four components: EE cage base, EE cage lid, standard cage feeder and standard cage lid.

water container at the same height as in standard cages. In addition, it includes 1-cm-diameter holes for maximum air exchange. The food and water container can be covered with a standard cage lid (see **Supplementary Data** for detailed plans for building the EE cage).

In our experiments[13], 12 female C57BL/6J mice at the age of weaning (4 weeks) were housed per EE cage. Female mice are preferred in experiments that use large EE cages because males can show territorial behavior and aggression, leading to stress that can affect behavioral results[22,23]. Nevertheless, several EE studies have used males without reporting any signs of aggression in the mice[24–26]. The incidence of fighting in males can be minimized if littermates are housed together immediately after weaning. As a control group, 12 female C57BL/6J mice at the age of weaning (4 weeks) were housed four per cage in standard polysulfone cages (36.5 × 20.7 × 14 cm) with bedding material, food and water *ad libitum*, and without any enrichment items.

We prefer the use of running wheels with a solid closed plastic floor (**Fig. 3a**) to standard metal wheels with rods. Previous studies have shown that mice have a strong preference for wheels with closed floors[27]. In addition, running wheels with closed floors are considered safer, as mice can injure their legs when running in open grids.

A few studies in rodents have provided different food items as a form of enrichment, such as cheese, crackers, peanuts and apples[7,28]. However, this approach is often considered to interfere with experimental setups that monitor metabolic or other parameters that could be influenced by changes in body weight. An alternative is to spread small food pellets throughout the bedding material to encourage foraging behavior.

Different types of nesting material can be used in EE experiments, such as paper towels, grass, paper strips, wood-wool and tissues. The advantage of using commercial nesting material such as pressed cotton squares (Nestlets; see EQUIPMENT list) is that they are pathogen free, noningestible, inert and nonirritating. Whatever nesting material is chosen should be autoclaved before use.

It has been shown that mice prefer paper-based nest boxes to plastic ones[29] (**Fig. 3b**). Mice can manipulate a paper-based box (they usually shred part of the boxes and even open new holes) and change its position because of its low weight. In contrast, plastic nest boxes are more rigid and always stay in the same position. However, a plastic nest box provides an additional complex three-dimensional environment; in our experiments, even in the presence of a paper-based box, mice also use the plastic nest as a shelter, suggesting that both types of nest boxes can be combined in the EE design. In addition, a red transparent plastic hammock (Mplex; see EQUIPMENT list) can also be used to enrich the cage[30]. It can be placed on the cage floor or hung from the cage feeder (**Fig. 3c**).

The use of plastic tubes as surrogate burrows is very common in EE studies. Mice appear to gain a great sense of security in these tunnels, probably because of their preference to retain contact with surfaces and to avoid open spaces (thigmotaxis). We use opaque polyvinyl chloride (PVC) tubes of 5-cm internal diameter (**Fig. 3d**), that can be assembled in different ways, such as by forming an L or a T shape.

One of the factors that consistently varies among studies is the duration of enrichment. One study has shown that a minimum enrichment period of 4 weeks is necessary to induce a behavioral effect in the open-field habituation task[31]. We have shown that an enrichment period of 6 weeks is sufficient to induce a robust anxiolytic-like effect in a battery of three anxiety tests[13]. Preliminary studies are recommended to determine the optimal enrichment period necessary to induce the desired effect.

Further reading

To better understand how to provide the right environment to address mouse species-specific needs, see reference 32 (available online at http://labanimals.awionline.org/pubs/cq02/Cq-mice.html).

For current information on EE products and suppliers, see http://www.nal.usda.gov/awic/enrichment/suppliers.htm and http://guide.labanimal.com/guide/product34.jsp?a=2&b=52480.

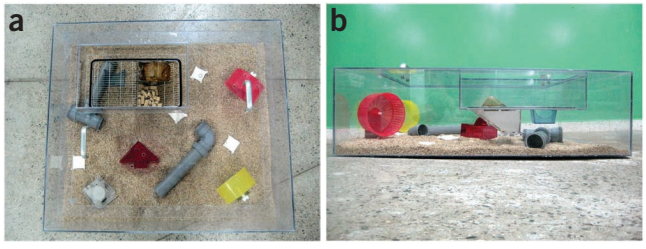

Figure 2 | Picture of the EE cage. (**a**) Top view of the EE cage. The standard cage lid was released to show the feeder. (**b**) Side view of the EE cage. Note the EE cage lid is specially designed to keep the feeder at the same height as in a standard mouse cage.

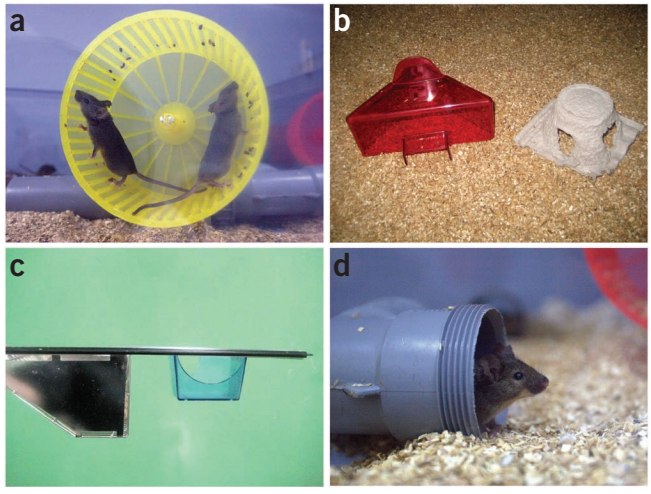

Figure 3 | Enrichment objects. (**a**) Mice in a running wheel with a solid closed plastic floor. (**b**) Red transparent plastic nest box and paper-based nest box. (**c**) Red transparent polycarbonate hammock hung from the cage feeder. (**d**) Mouse in a plastic tube.

MATERIALS

REAGENTS

- Mice (see REAGENT SETUP) ! CAUTION All experiments must be conducted in accordance with appropriate guidelines and regulations of the relevant authorities.
- Bedding material (such as Teklad Sani Chips; Harlan Laboratories)
- Ready-for-use disinfectant spray (such as Pharmacidal; Biological Industries, cat. no. IC-110100)
- Dishwashing detergent for cleaning plastic EE cage items (unscented)

EQUIPMENT

- EE cage (see EQUIPMENT SETUP)
- Rat standard cage (such as type IV Makrolon cage; Tecniplast)
- Mouse standard cage feeder (such as the one supplied with the type II Makrolon cage; Tecniplast)
- Mouse standard cage lid (such as the one supplied with the type II Makrolon cage; Tecniplast)
- Plastic tubes (such as PVC tubes; see EQUIPMENT SETUP)
- Running wheels
- Red transparent plastic nest box (Mouse House; Tecniplast)
- Paper-based nest box (Refuge; Otto Environmental, cat. no. KXKA-2450-087; see EQUIPMENT SETUP)
- Red transparent polycarbonate hammock (Mplex; Otto Environmental, cat. no. OEMPLEX; see EQUIPMENT SETUP)
- Wood blocks (see EQUIPMENT SETUP)
- Pressed cotton squares (Nestlets; Ancare, cat. no. NES3600; see EQUIPMENT SETUP)

REAGENT SETUP

Mice In our experiments, both EE and standard housed mice were maintained in a temperature-controlled room (22 °C ± 1 °C) on a reverse 12-h light-dark cycle. Food and water were given *ad libitum*.

EQUIPMENT SETUP

Cleaning and sterilization Before starting an experiment, all enrichment items should be disinfected or sterilized. In our laboratory, the EE cage and plastic objects are cleaned with water and detergent and disinfected using a ready-for-use disinfectant solution. Once inside the animal facility, the plastic objects should be washed once a week. We recommend the use of two or more sets of the same enrichment objects. Special attention should be paid to the washing of plastic tubes, as mice tend to urinate in the tubes and urine ammonia accumulates inside. In high concentrations, urine ammonia can be very stressful for mice. The pressed cotton squares, paper-based nesting box and wood blocks are sterilized by autoclaving and replaced with new ones once a week.

PROCEDURE

Experimental setup ● TIMING 5–10 min per EE cage

1| Place a clean and disinfected EE cage in an appropriate experimental room.

▲ CRITICAL STEP Keep the control group (mice housed in standard cages) in the same room and at the same distance from the light source as the EE cage for the entire experimental period.

? TROUBLESHOOTING

2| Fill the EE cage with autoclaved bedding material to a height of 2–3 cm.

3| Distribute enrichment items in the cage. We start with a defined set of objects (**Fig. 2**) that includes two running wheels, a paper-based nest box, a red transparent plastic nest box, PVC tubes and nesting material (four squares of Nestlets per 12 mice).

? TROUBLESHOOTING

4| Transfer 12 mice into the cage and cover the cage with the EE cage lid. Fill a standard cage feeder with food and water, place it on the feeder holder and cover with a standard cage lid (see **Fig. 1b**).

▲ CRITICAL STEP If the animals are purchased from an external company or transferred from a different animal facility, allow habituation to the experimental room for at least 1 week before starting the EE paradigm.

! CAUTION Individuals working with mice should avoid the use of personal-care products with strange odors, such as perfumes and deodorants, as they can produce stress responses in the animals. Similarly, change clothing and wash hands after handling predator species such as rats and cats to avoid causing fear reactions in the mice.

Cage cleaning and enrichment renew ● TIMING 10–15 min per EE cage

5| With the aid of the handles, uncover the EE cage and set the lid aside. Transfer the paper-based nest box and some of the shredded nesting material into a standard rat cage filled with bedding material. Thereafter, temporarily transfer the mice into the rat cage and cover it with its lid.

▲ CRITICAL STEP Ensure that both the EE and standard control cages are cleaned by the same person. Handling of animals by different persons could have undesirable effects on the experimental results and this variable should be controlled.

6| Remove all the enrichment objects from the cage, and using a plastic dustpan (which should be used only for this purpose), remove the bedding material from the cage (we recommend leaving a small amount of used bedding material in the cage to maintain familiar olfactory cues). There is no need to wash the EE cage with detergents until the end of the experiment.

7| Wash the plastic objects and let them dry.

8| Fill the EE cage with new autoclaved bedding material to a height of 2–3 cm.

9| Distribute clean enrichment objects in a different order for cognitive stimulation with respect to the formation of spatial maps. Add a new enrichment item such as the red transparent polycarbonate hammock or a wood block to increase the sense of novelty. Replace the nesting material and the paper-based nest box with a new one.
? TROUBLESHOOTING

10| Transfer the mice from the rat cage to the EE cage. We recommend keeping the same bedding material in the rat cage over the course of the experiment to reduce any novel context-induced stress in subsequent cage cleanings.

11| Cover the EE cage with its lid, turning the food and water side 180°, to further increase cognitive stimulation and the sense of novelty.

12| Repeat Steps 5–11 once a week.

● TIMING
Steps 1–4, Experimental setup: 5–10 min per EE cage
Steps 5–12, Cage cleaning and enrichment renew: 10–15 min per EE cage

The duration of the entire enrichment program proposed in this protocol is 6 weeks.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1.**

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	Unavoidable audible or ultrasonic noise in the experimental room	Pressure hoses, running taps, computer monitors or alarms in the experimental room	Use a masking background noise such as white noise or a radio playing softly
3	Mice do not carry out enough nest-building behavior	Inappropriate mouse strain	Change the strain if possible. C57BL/6 mice are generally reliable nesters
		Inadequate nesting material	Change or combine the Nestlets with other nesting materials such as straw, shredded paper or paper tissue
	Mouse behavior cannot be observed	The enrichment objects are opaque	Use red transparent enrichment objects
1, 9	Poor reproducibility of experimental outcomes	The order and timing of the enrichment renewal was changed	Keep the order and timing of enrichment renewal constant
		Stressors in the experimental room	Carefully control husbandry conditions (noise, lighting, temperature, humidity)

ANTICIPATED RESULTS

Environmental enrichment for mice is an experimental paradigm applicable to many research areas. It has proven to be beneficial for the psychological and physical well-being of animals and is an excellent tool for studying gene-environment interactions. Our experience has shown that animals housed under enriched conditions show reduced levels of anxiety-like behavior[13] in classical anxiety tests such as the elevated plus maze[33] and the light-dark transfer test[34]. In the elevated plus maze test, the time spent in the open arms and the percentage of open-arm entries are typically 1.5- to 2-fold increased by enrichment housing (measuring the percentage of open-arm entries may be particularly helpful to rule out the possibility that the increase in exploratory behavior results from an effect of housing conditions on general locomotor activity). In the light-dark transfer test, the time spent in the light zone and the number of entries into the light zone are typically 1.2- to 1.4-fold increased. These results may vary slightly, as both tests are very sensitive to many factors, such as strain, sex and the exact testing protocol. In addition to a decrease in anxiety-like behavior levels, the basal corticosterone level of EE mice is expected to be reduced by 75% (ref. 13). Generally, corticosterone levels as well as the parameters measured in the elevated plus maze test and the light-dark transfer test are normally distributed; hence parametric statistics such as Student's *t*-test and analysis of variance can be used for data analysis. If some parameters appear to be non-normally distributed, use nonparametric statistics such as the Mann-Whitney *U*-test. If the experimental design involves repeated exposures to the anxiety tests or repeated blood sampling for corticosterone measurement, a repeated-measures analysis of variance should be used.

Finally, as the protocol presented here uses mice as an animal model, we strongly recommend reading the *Guide for the Care and Use of Laboratory Animals*[35], as well as reference 23, for the proper care of animals during the experiment.

Note: Supplementary information is available via the HTML version of this article.

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