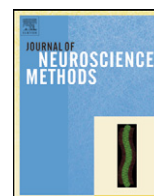




Contents lists available at ScienceDirect

## Journal of Neuroscience Methods

journal homepage: [www.elsevier.com/locate/jneumeth](http://www.elsevier.com/locate/jneumeth)

# A comparison of learning and memory characteristics of young and middle-aged wild-type mice in the IntelliCage

Annis O. Mehan\*, Adrian Wyss, Henry Rieger, M. Hasan Mohajeri

DSM Nutritional Products Ltd., R&amp;D Human Nutrition and Health, P.O. Box 2676, CH-4002 Basel, Switzerland

## ARTICLE INFO

## Article history:

Received 19 December 2008

Received in revised form 13 February 2009

Accepted 24 February 2009

## Keywords:

Learning

Memory

Cognition

Instinct

Behaviour

## ABSTRACT

We have tested the cognitive abilities of young (2.5 months) and middle-aged (14 months) wild-type C57Bl/6J mice in the IntelliCage, which enables automated monitoring of spontaneous and learning behaviour in a homecage-like environment. No differences were observed either in circadian activity or in performance in the novelty-induced exploration test, but middle-aged mice exhibited decreased exploratory activity overall. In the place learning test module, when mice were free to explore all corners without any negative reinforcement, young mice tended not to learn the task and performed less effectively than the middle-aged group. However, when an air-puff was administered as negative reinforcement following visits to an incorrect corner, young mice learned the task significantly better than middle-aged mice throughout the test period. Our data show that, in freely moving mice, the motivational cues for learning and retrieval of memory are age-dependent and dramatically influence learning and memory performance. Furthermore, the data reported here represent a step towards optimised cognitive test protocols when comparing young and middle-aged mice.

© 2009 Elsevier B.V. All rights reserved.

## 1. Introduction

In the field of cognitive neuroscience, there is a growing need for robust testing protocols to evaluate the cognitive performance of model organisms, especially rodents (Wahlsten et al., 2003). In order to draw meaningful conclusions about the effects of a pharmaceutical intervention or genetic manipulation, it is advisable to examine the animals in several tests (see Crawley and Paylor, 1997; Wolfer and Lipp, 2000). Historically, cognitive performance of rodents has been examined employing manifold protocols each requiring handling, training and testing the animals in a special test apparatus and arena. The reliability of such behavioural measurements across laboratories can vary considerably, especially with respect to spontaneous behaviours, as shown in a seminal study comparing data obtained by fully standardised test protocols (Crabbe et al., 1999). Subtly different handling by humans has been identified as the main source of inter-laboratory variation (Chesler et al., 2002a,b) since human behaviour cannot be standardised. Furthermore, cognitive behavioural testing of mice in several successively performed manual or semi-automated tests is a tedious job for the experimenters, and effects of sequential exposure to tests must be monitored and analysed (Võikar et al.,

2005). Finally, the use of severely adverse punishment (e.g. electric foot shocks) for studies on memory and learning bear the risk of confounding effects of non-physiological stress levels (Barrett et al., 1973), while stress-induced release of glucocorticoid hormones influences spatially orientated cognitive performance (Brinks et al., 2007), memory consolidation and memory retrieval (Roosendaal, 2002). Therefore, automated human-independent test procedures are needed to discover subtle, yet reliable, effects of mutations, treatments and aging (Tecott and Nestler, 2004).

We have compared the behaviour of young and moderately aged (middle-aged) mice in the IntelliCage system, which enables automatic monitoring of the mice's behaviour over an extended period of time in a homecage-like environment in which the mice are kept in social groups. Earlier studies in mice kept in groups and living in outdoor, semi-naturalistic environments revealed that significant behavioural differences resulting from targeted mutations could be recognised easily despite substantial environmental variation and social interactions between animals (Vyssotski et al., 2002; Lewejohann et al., 2004). The IntelliCage was initially validated for testing experimental animals in cognitive and motivational paradigms without overt stress caused by social isolation and different test environments (Galsworthy et al., 2005; Lipp, 2005; Knapska et al., 2006; Lipp et al., 2006; Onishchenko et al., 2007; Viosca et al., 2009). Moreover, the IntelliCage system discriminated rapidly between animals with varying degrees of hippocampal damage housed together with control mice (Lipp et al., 2004), indicating that the IntelliCage is suitable for testing hippocampal-dependent behaviour.

\* Corresponding author at: DSM Nutritional Products Ltd., R&D Human Nutrition and Health, Building 205/216a, P.O. Box 2676, CH-4002 Basel, Switzerland. Tel.: +41 61 815 8312; fax: +41 61 815 8740.

E-mail address: [annis.mayne-mechan@dsm.com](mailto:annis.mayne-mechan@dsm.com) (A.O. Mehan).

This paper is aimed at identification of the effect of age on activity levels and learning performance by comparing 12 middle-aged and 12 young female C57Bl/6J mice. Each IntelliCage contained equal numbers of animals from both test groups, thus ensuring highly standardised housing and test conditions. Female mice were used for this study because they are more suitable for aging studies as, unlike males, they can be housed in peer groups for extended time periods and can be introduced into other groups. We use the term “middle-aged” here to denote mice aged between 10 and 14 months (Francia et al., 2006; Harrison, 2009), while different substrains had to be used for economic reasons. We show here that young mice were either indistinguishable from middle-aged mice or performed less effectively in a learning task without negative reinforcement. After the introduction of an air-puff punishment for choosing the incorrect corner, however, young mice quickly learned the task and were able to retrieve the information until the end of the testing period.

## 2. Materials and methods

### 2.1. Animals and husbandry

Twelve middle-aged- and 12 young adult-female C57Bl/6J mice were used for this study. The middle-aged mice (“A.C”; bred at the University of Zürich, Zürich, Switzerland) were 14 months of age at the start of the experimental phase, while the young mice (“Y.C”; Elevage Janvier, Le Genest-Saint Isle, France) were ten weeks of age. Mice were housed in the test facility for six weeks prior to the start of behavioural testing.

All experiments were performed in accordance with the Swiss Federal Act on Animal Protection and Swiss Animal Protection Ordinance and as approved by the cantonal ethics committee.

### 2.2. Acclimatisation period

After four days’ initial acclimatisation, during which time middle-aged and young mice were housed in separate groups, mice were randomly assigned to homecages (Type IV; 33 cm × 55 cm × 20 cm), containing standard sawdust bedding, such that each cage included equal numbers of mice belonging to each experimental group (total:  $n = 12$  per cage). Mice had free access to food (ssniff R/M-Haltung, extrudate; ssniff, Soest, Germany) and water throughout the acclimatisation period and were housed under reversed-light conditions (lights on: 22:00–10:00), in an ambient temperature of  $22 \pm 1$  °C and 50–60% relative humidity.

### 2.2.1. Transponder implantation

After the initial acclimatisation period, all mice were implanted with microtransponders (implantable RF tags; DATAMARS SA, Bedano/Lugano, Switzerland). Implantation was performed under light anaesthesia, which was initiated by administering 4.5% isoflurane (DeltaSelect GmbH, Dreieich, Germany) in oxygen, then maintained at 2.2–2.4% isoflurane. The transponders were then subcutaneously injected into the scruff of the neck using the supplied disposable syringes and identification of each mouse was performed using a hand-held electronic reader (MINI MAX II; DATAMARS SA). The entire transponder implantation procedure lasted approximately 2 min starting from initiation of anaesthesia and being completed once the mice were fully awake.

### 2.3. Experimental phase

Following the acclimatisation period, mice were transferred to the IntelliCages ( $n = 12$  per cage). The sawdust bedding used in the IntelliCages was of a coarser consistency, to minimise dust and small particles being carried into the recording chambers. Mice

had free access to food, while water access was limited during certain modules of the experimental phase (see Test Schedule, below). Lighting, temperature and humidity conditions were maintained as during the acclimatisation period. Mice were thus maintained in the IntelliCages for a total of five weeks during the experimental phase.

### 2.3.1. IntelliCages

The IntelliCage (NewBehavior AG, Zürich, Switzerland) enables automated monitoring of spontaneous and learning behaviour of mice in a homecage-like environment. Each IntelliCage (37.5 cm × 55 cm × 20.5 cm) houses four recording (operant) chambers, which fit into the corners of the cage, each covering a 15 cm × 15 cm × 21 cm right-angled triangular area of floor space (Fig. 1). In-cage antennae enable automated monitoring of each individual mouse’s corner visits, while photo-beams within each corner enable automated recording of individual nose-pokes. Licks of the water bottle spouts are recorded electrically by means of a very weak current passing through the bottle spouts. Four triangular mouse shelters were placed in the centre of each cage, above which was situated a food hopper, enabling *ad libitum* access to food. For further details of the IntelliCage set-up, see Galsworthy et al. (2005), Knapska et al. (2006), Onishchenko et al. (2007) and www.newbehavior.com.

### 2.4. Test schedule

In this study, a number of different test modules were utilised in an attempt to gather information concerning general exploratory- and learning-behaviour. In addition to analysis of test-group differences in behaviour, the behaviour of each individual mouse within- and between-modules was assessed. During each module, mice were monitored for their licking (drinking) behaviour; any mouse which did not perform any licks for more than 48 h was removed from its IntelliCage and excluded from the remainder of the study. Thus the following mice were excluded: Adaptation: A.C,  $n = 1$ ; Place learning 1: A.C,  $n = 1$ ; Place learning 2: Y.C,  $n = 2$ ; Reversal of place learning 2: Y.C,  $n = 3$ .

New modules were started in the morning, prior to the lights being turned off and the start of the active phase (i.e. at 09:00).

### 2.4.1. Adaptation/spontaneous exploration

Mice were allowed to explore the cages freely for six days and thus to familiarise themselves with their surroundings. All corners

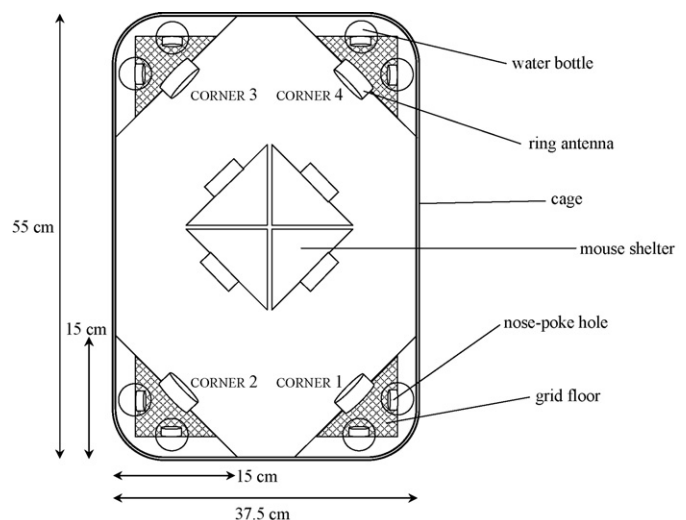


Fig. 1. Diagram of the IntelliCage, showing the four recording chambers, each including a recording antenna, two nose-poke holes and two water bottles.

165 were accessible to all mice, meaning that all doors were open and  
166 all water bottle spouts could be reached. Data collected during  
167 this phase comprised number and duration of corner visits, nose-  
168 pokes and licks. The number of corner visits provided a measure of  
169 exploratory activity and any preferences for particular corners of  
170 each individual mouse could be assessed.

#### 171 2.4.2. Nose-poke adaptation

172 During this module (two days), all doors were initially closed.  
173 The aim of this module was that the mice had to learn to perform  
174 a nose-poke in order to open a door (a simple operant response)  
175 and to reach a water bottle. Following a successful nose-poke, a  
176 door remained open for 7 s and mice could open a door repeat-  
177 edly during a single corner visit. Data collected comprised the same  
178 parameters as during the acclimatisation phase; in particular, the  
179 least-preferred corner of each individual mouse was noted for pro-  
180 gramming the next module.

#### 181 2.4.3. Place learning I

182 In this module (five days), the least-preferred corner of each  
183 individual mouse, as recorded during nose-poke adaptation, was  
184 programmed to be “accessible” to that particular mouse, while  
185 all other corners were “inaccessible”. As such, all mice could still  
186 enter and nose-poke within all corners, but nose-pokes in only the  
187 selected corner would trigger the doors to open and the water bot-  
188 tle to be reached (“correct” corner). Nose-pokes in all other corners  
189 would not trigger opening of the motorised doors (“incorrect” cor-  
190 ners). Thus, the ability of each mouse to learn to find the “correct”  
191 (rewarded) corner provided a simple measure of place learning.  
192 Data collected included the error rate for each mouse, calculated  
193 as (number of “incorrect” corner visits/total number of corner vis-  
194 its)  $\times 100$ .

#### 195 2.4.4. Reversal of place learning I

196 During this module (four days), each mouse again had free access  
197 to one “correct” corner while all other corners were “incorrect” and  
198 did not permit access to the water bottles. However, a different “cor-  
199 rect” corner was programmed for each mouse, as that which was  
200 diagonally opposite to the “correct” corner assigned during place  
201 learning I. Exploratory activity and error rates were subsequently  
202 analysed, the latter indicating the rate at which each mouse learned  
203 the new “correct” corner.

#### 204 2.4.5. Behavioural extinction

205 In order to extinguish previously learned behaviours, mice were  
206 allowed free exploration of the IntelliCages for ten days, during  
207 which time all corners and water bottle spouts were accessible to  
208 all mice.

#### 209 2.4.6. Novelty-induced exploration

210 An object (1.5 ml eppendorf tube) was suspended below the grid  
211 floor, using a small piece of wire, in a randomly selected corner of  
212 each IntelliCage. All mice had free access to all corners and “novel  
213 object attractiveness” was assessed by calculating the percentage of  
214 visits to the corner containing the object [(visits to “novel object  
215 corner”/total number of corner visits)  $\times 100$ ] throughout 3 h following  
216 object presentation and comparing the values with those obtained  
217 during the 3 h preceding presentation of the object.

#### 218 2.4.7. Place learning II—negative reinforcement

219 In order to further investigate place learning behaviour, mice  
220 were again tested in this module (three days). The least-preferred  
221 corner, as determined during the behavioural extinction phase, was  
222 designated as the “correct” (rewarded) corner for each individual  
223 mouse. However, in contrast to place learning I, visits to “incorrect”  
224 corners resulted in aversive stimulation, in the form of an air-puff of

1–2 bar pressure for 1 s. In this manner, the cost of exploration was  
increased, as a penalty was now incurred for visiting an “incorrect”  
corner.

#### 228 2.4.8. Reversal of place learning II—negative reinforcement

229 In this module (three days), the “correct” corner was designated  
230 as that which was diagonally opposite to the “correct” corner during  
231 place learning II. Visits to “incorrect” corners were again subject to  
232 negative reinforcement (an air-puff).

#### 233 2.5. Statistics

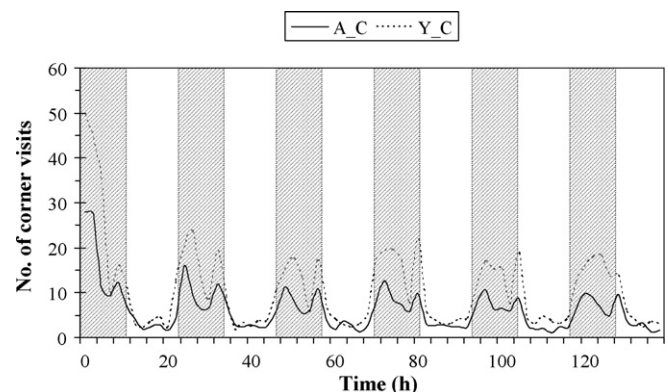
234 Comparisons between groups were performed using repeated  
235 measures analysis of variance (ANOVA) or, where applicable, inde-  
236 pendent samples *t*-tests (SPSS 13.0 EN R4; SPSS Inc., Chicago, IL,  
237 USA). In all instances where numbers of licks per corner visit were  
238 analysed, only visits where licks were recorded were taken into con-  
239 sideration. Data are reported as mean  $\pm$  standard error of the mean  
240 (S.E.M.).

### 241 3. Results

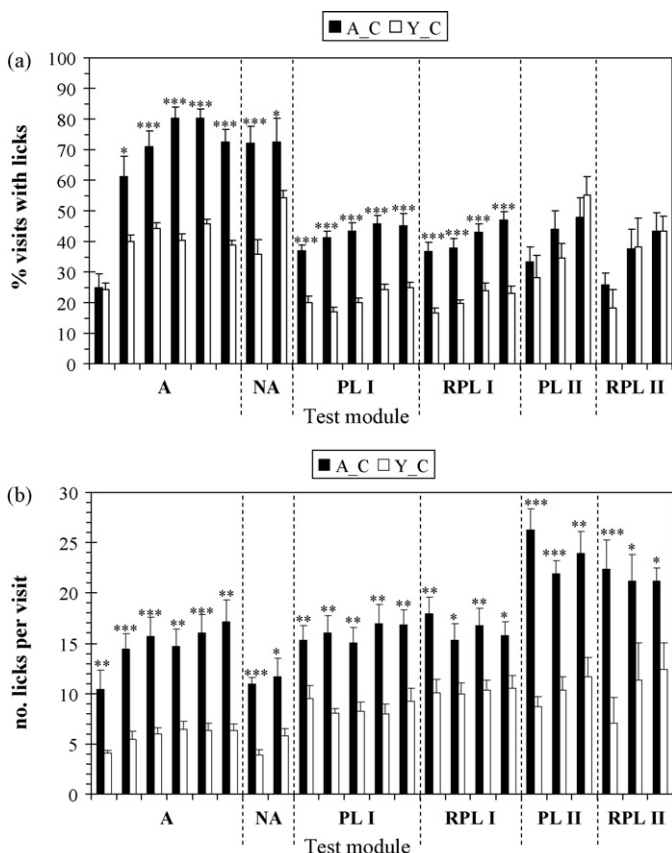
#### 242 3.1. Adaptation/spontaneous exploration

243 During this first phase of the study, where all mice had free  
244 access to all areas of the cage, both groups demonstrated circadian  
245 rhythm-related patterns of exploratory behaviour (significant  
246 effect of Time:  $F(69, 1449) = 45.07$ ,  $p < 0.001$ ; Fig. 2). Middle-aged  
247 mice demonstrated lower exploratory activity, i.e. 40–60% fewer  
248 corner visits than young mice, during the adaptation period  
249 (significant effect of Group:  $F(1, 21) = 38.19$ ,  $p < 0.001$ ; significant  
250 interaction effect of Time  $\times$  Group:  $F(69, 1449) = 5.04$ ,  $p < 0.001$ ).  
251 The overall group corner preferences, as observed over the entire  
252 six-day module, were as follows: A.C:  $24.75 \pm 1.63\%$ ,  $23.19 \pm 0.96\%$ ,  
253  $25.06 \pm 1.30\%$ ,  $27.00 \pm 1.26\%$  and Y.C:  $27.17 \pm 0.49\%$ ,  $24.14 \pm 0.60\%$ ,  
254  $24.28 \pm 0.78\%$ ,  $24.42 \pm 0.89\%$  (corners 1, 2, 3 and 4, respectively).

255 Although the middle-aged animals performed fewer corner vis-  
256 its than the young animals, the former performed significantly  
257 greater numbers of licks per visit (significant effect of Group:  
258  $F(1, 21) = 33.49$ ,  $p < 0.001$ ; Fig. 3b) and approximately 60–80%  
259 of corner visits included drinking behaviour. In contrast, almost two-  
260 thirds of corner visits performed by the young mice did not include  
261 any drinking behaviour (Fig. 3a). Furthermore, there was an overall  
262 increase in drinking frequency in both groups, with each suc-  
263 cessive day of the adaptation period (significant effect of Time:  
264  $F(5, 105) = 7.16$ ,  $p < 0.001$ ; Fig. 3b).



265 Fig. 2. Acclimatisation phase. Data are shown as the response per test group  
266 throughout the acclimatisation phase, as calculated per 2 h time-bin (mean  $\pm$  S.E.M.,  
267 middle-aged mice; A.C:  $n = 11$ , young mice; Y.C:  $n = 12$ ). Shaded areas depict the dark  
268 (i.e. active) phase of the light/dark cycle (10:00–22:00).



**Fig. 3.** Drinking frequency. (a) Percentage of visits including licks, (b) number of licks per visit with licks. Data are shown as the response per test group per day of the separate test modules (mean  $\pm$  S.E.M.), only taking into account activity recorded during the active phase of the light/dark cycle. The graphs are divided up into test modules: A: adaptation (A.C:  $n=11$ , Y.C:  $n=12$ ), NA: nose-poke adaptation (A.C:  $n=11$ , Y.C:  $n=12$ ), PL I: place learning I (A.C:  $n=10$ , Y.C:  $n=12$ ), RPL I: reversal of place learning I (A.C:  $n=10$ , Y.C:  $n=12$ ), PL II: place learning II (A.C:  $n=10$ , Y.C:  $n=10$ ), RPL II: reversal of place learning II (A.C:  $n=10$ , Y.C:  $n=7$ ). Significant differences between groups are denoted as \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

### 3.2. Nose-poke adaptation

During this phase, mice could only drink after performing a nose-poke to open the doors in any corner. All animals successfully learned to nose-poke and thus to reach the water bottles. Again, middle-aged mice performed fewer corner visits than young mice on both test days, while both groups performed greater numbers of corner visits on Day 2, compared with Day 1 (Day 1: A.C:  $44.18 \pm 3.41$ , Y.C:  $50.42 \pm 7.74$ ; Day 2: A.C:  $54.18 \pm 3.86$ , Y.C:  $68.83 \pm 8.40$ ). However, while there was a significant difference in numbers of corner visits between the two test days (significant effect of Time:  $F(1,21)=10.50$ ,  $p < 0.01$ ), there was no difference between the test groups (effect of Group:  $F(1,21)=1.70$ ,  $p=0.21$ ; interaction effect of Time  $\times$  Group:  $F(1,21)=0.92$ ,  $p=0.35$ ).

The least-preferred corner of each individual mouse was selected for the subsequent place learning module, similar numbers of mice being assigned to each corner within each cage. The overall group corner preferences, as observed over the entire two-day module, were as follows: A.C:  $24.35 \pm 2.13\%$ ,  $22.40 \pm 1.50\%$ ,  $23.55 \pm 1.87\%$ ,  $29.70 \pm 2.40\%$  and Y.C:  $25.90 \pm 2.24\%$ ,  $24.37 \pm 1.67\%$ ,  $25.14 \pm 2.33\%$ ,  $24.59 \pm 2.29\%$  (corners 1, 2, 3 and 4, respectively).

Drinking frequency was significantly higher in middle-aged mice, as demonstrated by a higher percentage of visits including licks (significant effect of Group:  $F(1,21)=15.18$ ,  $p < 0.01$ ; Fig. 3a) and a higher number of licks per visit (significant effect of Group:  $F(1,21)=27.40$ ,  $p < 0.001$ ; Fig. 3b), compared with young mice.

### 3.3. Place learning I

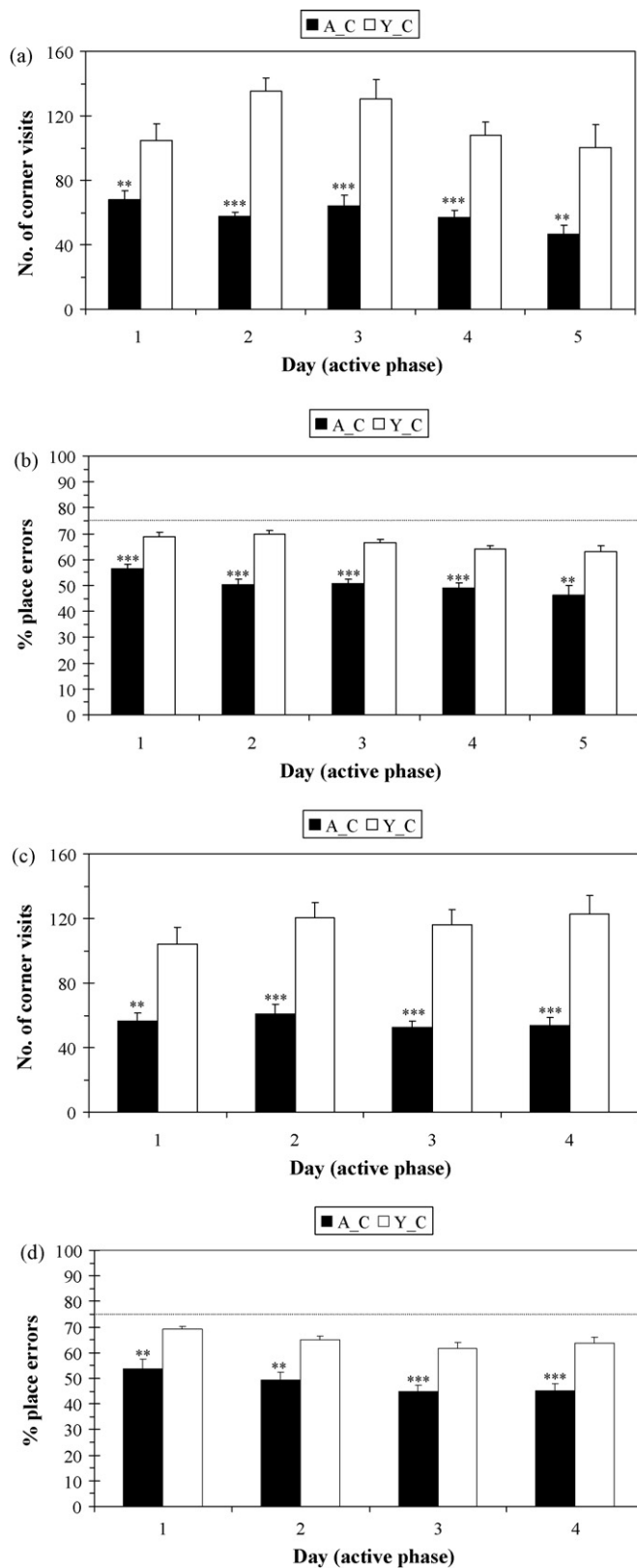
Each mouse was assigned to one "correct" corner, in which it was able to drink, while the other three corners were "incorrect" and thus access to the water bottles was blocked. As seen during the adaptation phase, the number of corner visits performed by the middle-aged mice was approximately 35–58% lower than by the young animals (Fig. 4a), resulting in significant overall differences between groups (significant effect of Group:  $F(1,20)=32.91$ ,  $p < 0.001$ ; Days 1 and 5:  $p < 0.01$ , Days 2–4:  $p < 0.001$ ). In addition, there was a gradual decline in the number of corner visits performed by middle-aged mice, while the young mice performed a greater number of corner visits on Day 2, compared with on Day 1, followed by a decline on Days 3–5 (significant effect of Time:  $F(4,80)=5.24$ ,  $p < 0.01$ ; significant interaction effect of Time  $\times$  Group:  $F(4,80)=3.28$ ,  $p < 0.05$ ). Both groups showed an improvement in performance over time (significant effect of Time:  $F(4,80)=6.93$ ,  $p < 0.001$ ), but the middle-aged animals displayed a consistently lower error rate, compared to the young group, on all test days (significant effect of Group:  $F(1,20)=74.23$ ,  $p < 0.001$ ; Days 1–4:  $p < 0.001$ , Day 5:  $p < 0.01$ ; Fig. 4b).

The middle-aged mice drank with a significantly higher frequency, performing greater numbers of visits including licks (significant effect of Group:  $F(1,20)=72.54$ ,  $p < 0.001$ ; Fig. 3a) and greater numbers of licks per corner visit (significant effect of Group:  $F(1,20)=29.65$ ,  $p < 0.001$ ; Fig. 3b), compared with the young group, on all test days.

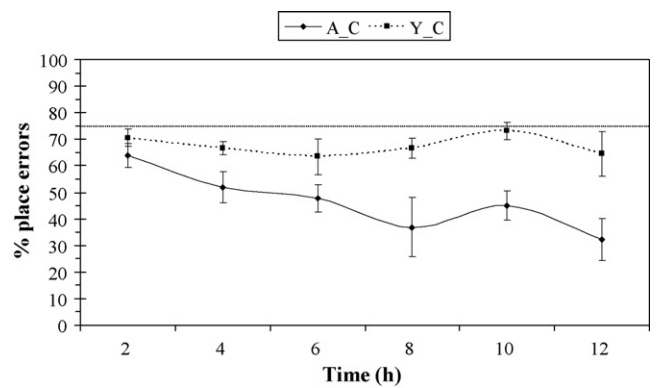
### 3.4. Reversal of place learning I

In the reversal of place learning module, each mouse was assigned a new "correct" corner, which was diagonally opposite to the "correct" corner in the place learning module. As observed in the previous modules, the young mice demonstrated significantly greater exploratory activity than the middle-aged mice, performing greater numbers of corner visits on all test days (significant effect of Group:  $F(1,20)=33.81$ ,  $p < 0.001$ ; Day 1:  $p < 0.01$ , Days 2–4:  $p < 0.001$ ; Fig. 4c). Moreover, significant differences in error rate between groups were observed on all test days, the discriminatory behaviour of the middle-aged mice again being significantly higher, i.e. error rate being lower, than that of the young mice (significant effect of Group:  $F(1,20)=35.64$ ,  $p < 0.001$ ; Days 1 and 2:  $p < 0.01$ , Days 3 and 4:  $p < 0.001$ ; Fig. 4d). While the error rate of both groups declined on successive days (significant effect of Time:  $F(3,60)=8.72$ ,  $p < 0.001$ ), there was little change in the overall exploratory behaviour of the mice, as the number of corner visits, within groups, remained consistent from day to day (effect of Time:  $F(3,60)=1.82$ ,  $p=0.15$ ).

During the first day, a learning effect was seen over time in the middle-aged mice (Fig. 5), whereby the error rate decreased from  $63.89 \pm 4.45\%$ , during the first 2 h after the start of the module, to  $32.22 \pm 7.92\%$  after 12 h. This resulted in an overall significant effect of Time ( $F(5,100)=9.78$ ,  $p < 0.001$ ) and a significant interaction effect of Time  $\times$  Group ( $F(5,100)=2.50$ ,  $p < 0.05$ ). The middle-aged mice thus remained consistently well below the chance level (i.e. error rate below 75%), while the young group maintained an error rate of between 63% and 73% throughout the first 12 h of this module (significant effect of Group:  $F(1,20)=8.49$ ,  $p < 0.01$ ). Drinking frequency, as depicted by the number of visits including licks and the number of licks per corner visit, was again significantly higher in middle-aged mice, compared with young mice (significant effects of Group:  $F(1,20)=65.69$ ,  $p < 0.001$  (Fig. 3a) and  $F(1,20)=13.10$ ,  $p < 0.01$  (Fig. 3b), respectively).



**Fig. 4.** Place learning I: (a) number of corner visits, (b) error rate. Reversal of place learning I: (c) number of corner visits, (d) error rate. Data are shown as the response per test group per day of the separate test modules (mean  $\pm$  S.E.M., A.C:  $n = 10$ , Y.C:  $n = 12$ ), only taking into account activity recorded during the active phase of the light/dark cycle. In (b) and (d), the horizontal, dashed, line depicts the chance level (75%), whereby visits to three out of four corners results in an error. Significant differences between groups are denoted as \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .



**Fig. 5.** Reversal of place learning I: error rate. Data are shown as the average response per test group (mean  $\pm$  S.E.M., A.C:  $n = 10$ , Y.C:  $n = 12$ ), place errors being calculated per 2 h time-bin, over the first 12 h (active phase) of the test module. The horizontal, dashed, line depicts the chance level (75%), whereby visits to three out of four corners results in an error.

### 3.5. Novelty-induced exploration

Following ten days' behavioural extinction, mice were presented with an object in one corner of their cage, during the dark (active) phase. The number of visits to all corners during the 3 h prior to and 3 h following presentation of the object were recorded (Table 1). All animals visited the "novel object corner" more frequently during the 3 h after the object was presented, compared with the number of visits during the preceding 3 h, resulting in a significant effect of Time ( $F(1, 20) = 17.53, p < 0.001$ ). However, while the young mice performed greater numbers of corner visits than the middle-aged mice, both prior to and following object presentation, there was no statistically significant difference between groups (effect of Group:  $F(1, 20) = 0.68, p = 0.42$ , interaction effect of Time  $\times$  Group:  $F(1, 20) = 5.81, p = 0.83$ ). All other corners were visited at a lower and similar frequency by both groups (data not shown).

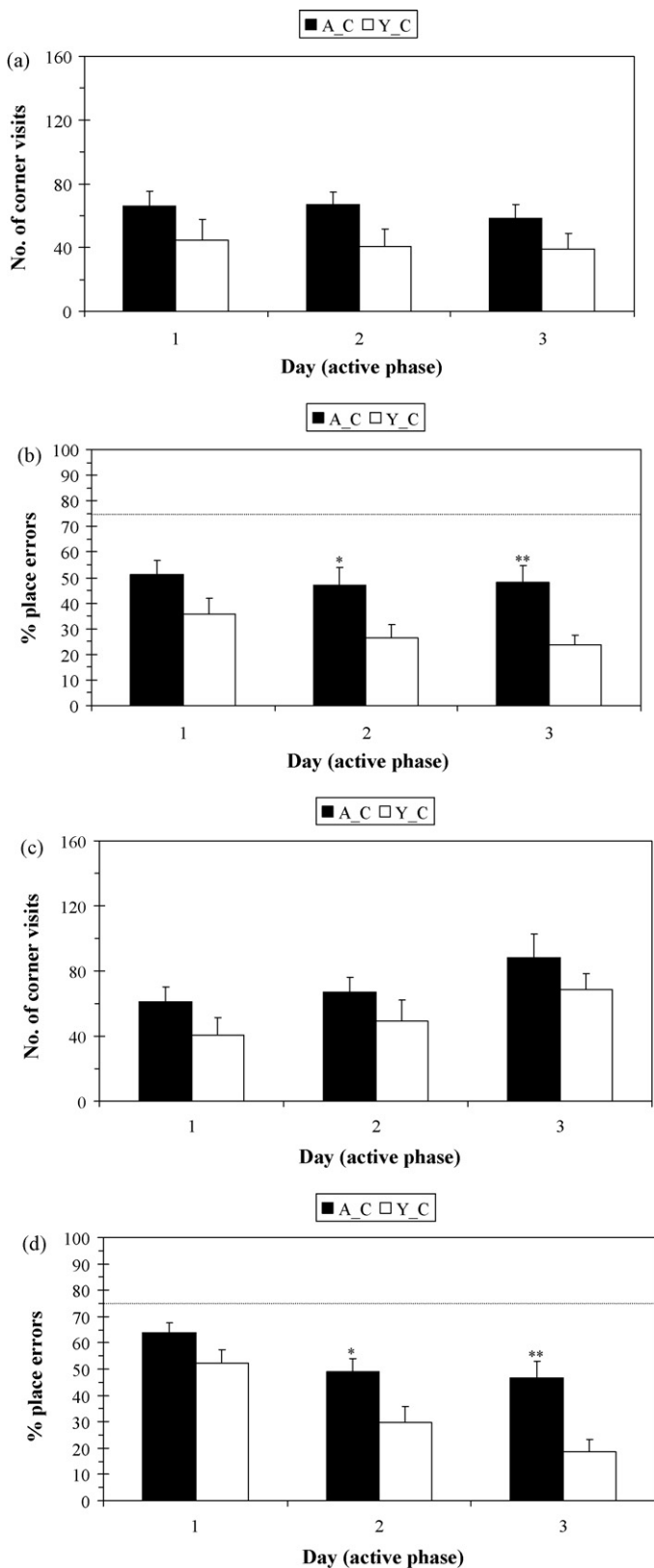
### 3.6. Place learning II—negative reinforcement

In order to further elucidate effects of age on learning and memory, place learning- and reversal of place learning-behaviour were again investigated with the addition of aversive stimulation in the form of an air-puff, when mice visited "incorrect" corners. Such negative reinforcement had a substantial effect on the behaviour of the young animals, as they performed fewer corner visits and demonstrated improved discriminatory behaviour compared with place learning I (compare Figs. 6a and 4a and Figs. 6b and 4b, respectively). In contrast, neither the number of corner visits nor the error rate of middle-aged mice differed markedly whether in the presence or absence of aversive stimulation. Although young mice performed fewer corner visits than middle-aged mice, following negative reinforcement, this effect was not statistically significant (effect of Group:  $F(1, 18) = 2.66, p = 0.12$ ; Fig. 6a), nor was there any difference in either group with respect to numbers of corner visits when compared between test days (no effect of Time:  $F(2, 36) = 1.91$ ,

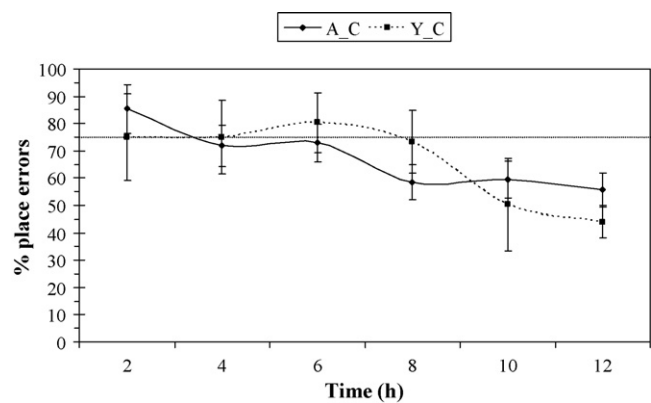
**Table 1**

Novelty-induced exploration: percentage of visits to Corner 1 (i.e. (visits to Corner 1/total number of corner visits)  $\times$  100), during the 3 h prior to (N0) and following (N1) commencement of the presentation of an object. Data are shown as mean  $\pm$  S.E.M. (middle-aged mice; A.C:  $n = 10$ , young mice; Y.C:  $n = 12$ ).

| Group | $n$ | Percentage of visits to Corner 1 (mean $\pm$ S.E.M.) |                  |                  |
|-------|-----|--|------------------|------------------|
|       |     | N0   | N1               | N1 - N0          |
| A.C   | 10  | 26.15 $\pm$ 4.74                                     | 39.13 $\pm$ 3.58 | 12.98 $\pm$ 4.92 |
| Y.C   | 12  | 28.82 $\pm$ 3.08                                     | 43.26 $\pm$ 3.50 | 14.44 $\pm$ 4.35 |



**Fig. 6.** Place learning II: (a) number of corner visits, (b) error rate. Reversal of place learning II: (c) number of corner visits, (d) error rate. Data are shown as the response per test group per day of the separate test modules (mean  $\pm$  S.E.M., A.C:  $n=10$ , Y.C:  $n=10$  (place learning II) and  $n=7$  (reversal of place learning II)), only taking into account activity recorded during the active phase of the light/dark cycle. In (b) and (d), the horizontal, dashed, line depicts the chance level (75%), whereby visits to three out of four corners results in an error. Significant differences between groups are denoted as \* $p < 0.05$  and \*\* $p < 0.01$ .



**Fig. 7.** Reversal of place learning II: error rate. Data are shown as the average response per test group (mean  $\pm$  S.E.M., A.C:  $n=10$ , Y.C:  $n=7$ ), place errors being calculated per 2 h time-bin, over the first 12 h (active phase) of the test module. The horizontal, dashed, line depicts the chance level (75%), whereby visits to three out of four corners results in an error.

$p=0.16$ ). However, in contrast to place learning I, the young mice demonstrated improved discriminatory behaviour, compared with the middle-aged group (significant effect of Group:  $F(1, 18)=6.59$ ,  $p < 0.05$ ; Day 2:  $p < 0.05$ , Day 3:  $p < 0.01$ ; Fig. 6b), while an improvement between test days in the young animals resulted in an overall significant effect of Time ( $F(2, 36)=6.53$ ,  $p < 0.01$ ).

Negative reinforcement also had a marked effect on the drinking frequency of young mice, the number of visits including licks being higher than in place learning I, while having little effect on the drinking frequency of middle-aged mice (no effect of Group:  $F(1, 18)=0.10$ ,  $p=0.76$ ; Fig. 3a). However, the number of licks per corner visit was noticeably higher in middle-aged mice in place learning II, than in place learning I, while little change was observed in young mice (significant effect of Group:  $F(1, 18)=65.80$ ,  $p < 0.001$ ; Fig. 3b).

### 3.7. Reversal of place learning II—negative reinforcement

Although there was little difference between young and middle-aged mice with regard to the number of corner visits on all test days (effect of Group:  $F(1, 15)=1.65$ ,  $p=0.22$ ; Fig. 6c), the learning performance of both groups improved with each subsequent day of testing (effect of Time:  $F(2, 30)=15.16$ ,  $p < 0.001$ ; Fig. 6d). As in the previous module (place learning II), middle-aged mice displayed a higher error rate than young mice on all test days (significant effect of Group:  $F(1, 15)=10.84$ ,  $p < 0.01$ ; Day 2:  $p < 0.05$ , Day 3:  $p < 0.01$ ; Fig. 6d), and in contrast to reversal of place learning I, where middle-aged mice demonstrated significantly higher levels of discriminatory behaviour (lower error rates) throughout the task (Fig. 4d).

The inclusion of negative reinforcement markedly altered the error rate over time (Fig. 7). In the learning paradigm without reinforcement, middle-aged mice demonstrated a learning effect over time, while the young mice maintained a fairly consistent error rate throughout the first few hours of the module (Fig. 5). In the current module, while the learning performance of both groups improved over time (significant effect of Time: ( $F(5, 75)=2.76$ ,  $p < 0.05$ ), there was no significant difference between young and middle-aged mice (no effect of Group:  $F(1, 15)=0.32$ ,  $p=0.58$  or interaction effect of Time  $\times$  Group:  $F(5, 75)=0.26$ ,  $p=0.93$ ). It should be noted, however, that only ten middle-aged animals and only seven young animals performed corner visits during the first 12 h of aversive stimulation, therefore within-group variance was high.

There was no difference between groups with regard to the number of visits including licks (no effect of Group:  $F(1, 15)=0.14$ ,

$p = 0.72$ ; Fig. 3a), while both groups demonstrated an increase with each successive test day (significant effect of Time:  $F(2, 30) = 10.15$ ,  $p < 0.001$ ). Middle-aged mice again performed greater numbers of licks per visit, compared with young mice (significant effect of Group:  $F(1, 15) = 20.14$ ,  $p < 0.001$ ; Fig. 3b) and, while the number of licks per visit increased over time in the young mice, this effect was not significant (no significant effect of Time:  $F(2, 30) = 0.50$ ,  $p = 0.61$ ).

#### 4. Discussion

The results of our study show that, in the IntelliCage system, the learning performance of mice depended on their motor abilities and young wild-type mice only learned efficiently when there was a cost associated with lack of learning behaviour. The IntelliCage provides a unique opportunity to test animals living in their social group; contrary to expectations, group housing and environmental enrichment of mice does not increase variability in conventional behavioural tests (Wolfer et al., 2004), and genetically dependent behavioural differences are readily apparent even in mice living outdoors under extremely variable conditions (Vyssotski et al., 2002; Lewejohann et al., 2004). Likewise, housing male mice in social isolation over prolonged periods changes their behavioural responses (Vöikar et al., 2005). Thus, tests without social deprivation are more sensitive for detecting alterations in activity and learning paradigms, because low stress levels increase activity scores, particularly during early phases of exposure to novel environments, and thus may reveal modest differences otherwise masked by the high stress levels associated with classical behavioural tests. In particular, stress-induced increases in glucocorticoid hormone levels can impact on spatially orientated cognitive performance (Brinks et al., 2007), memory consolidation and memory retrieval (Roozendaal, 2002). A second benefit of the IntelliCage is the opportunity to study concomitantly reward and avoidance paradigms (Knapska et al., 2006). Thirdly, the IntelliCage system reduces the signal-to-noise ratio in an unbiased, automated manner, thus eliminating the uncontrollable confounding effects caused by experimenters (Chesler et al., 2002a,b), variations in experimental set-up (Crabbe et al., 1999) and variable stress reactions of mice being exposed to a variety of stimuli and situations (Vöikar et al., 2005), as associated with the classical cognitive behavioural tests.

All mice adapted to the IntelliCage system and learned to nose-poke in order to access the water bottles. As expected, levels of exploratory activity were markedly higher in the young animals, when no aversive stimulation was imposed. Even though middle-aged mice were far less active than the young group, all mice exhibited typical circadian rhythms with nocturnal increases in activity. Moreover, all mice performed equally well in the novelty-induced exploration test, an example of exploratory learning behavior. When the animals were tested in the place learning paradigm without any punishment for the wrong choice, young mice did not distinguish between the rewarded corner (access to water) and corners without access to water and persisted in making numerous visits to the incorrect corners. To test the hypothesis that superfluous activity, i.e. strong locomotor activity of young mice, was responsible for their high error rate (Galsworthy et al., 2005), we trained and tested the same group of mice after an extended wash-out period in an identical protocol, but punished every visit to an incorrect corner with an air-puff. It should be stressed that these experimental conditions resemble the natural conditions more accurately because wrong choices had a negative consequence for the mice. Under these conditions, young mice learned the task much better than their middle-aged counterparts, a result that is in agreement with published data generated in classical behavioural testing (see Fukui et al., 2002; Singh et al., 2003;

Sumien et al., 2004; Baskerville et al., 2006). Furthermore, in previously published studies, the mechanisms of observed memory deficits in aged animals have been shown to include an alteration in expression of genes related to inflammation, oxidative stress and protein processing (see Forster et al., 1996; Verbitsky et al., 2004).

How mice react to a novel situation results from competition between an exploratory tendency, motivated by curiosity or boredom, and a withdrawal tendency that is motivated by fear (Lister, 1990). In other words, hereditary instincts to explore holes and dark areas as probable shelter places, lead mice to enter the IntelliCage corners to avoid exposure in an open arena, but the unknown corner might also be viewed as potentially dangerous and can, itself, arouse fear. Our results show that, without punishment, all mice explored the corners according to their activity level. From the start of the study, middle-aged mice were less active than their young counterparts. For example, during the adaptation phase, middle-aged mice performed 40–60% fewer corner visits per day than the young mice. Besides physiologically motivated visits (drinking), 63% of visits by young mice were made out of curiosity (without drinking), which is considered a normal type of behaviour for young adult mice. Conversely, 60% of visits performed by middle-aged mice included drinking behaviour. Having found the accessible water bottles, these mice drank with a significantly higher frequency during each visit, compared with young mice, indicating that they tried to minimise the extent of exploration (movement), thus avoiding high levels of energy expenditure. As such, corner visits by middle-aged mice were likely to be motivated by physiological needs, rather than purely for the purpose of exploration. Young mice, however, were more active and explored all corners with a frequency indicated by the chance level, because a wrong choice had no negative consequences. Since both groups demonstrated ability for place learning, and similar reactivity to novel objects, these differences cannot be attributed to age-dependent differences in attention, curiosity or basic learning abilities.

The use of negative reinforcement, by applying an air-puff at each incorrect visit, significantly improved the learning performance of young mice, while having little impact on that of middle-aged mice. Thus, the young mice had a lower error rate than middle-aged mice, an effect which reached statistical significance during the second day of the place learning task and persisted thereafter, and was in contrast to the behaviour observed in the absence of negative reinforcement. Although young mice performed far fewer corner visits under aversive stimulation, indicating a reluctance to explore, both middle-aged and young groups were able to learn the location of the rewarded corner, providing evidence of a normal ability for spatial learning. One explanation for the varying behavioural characteristics of young vs. middle-aged mice is that young mice weighed the aversive stimulus more strongly—when a cost was associated with exploration, the number of corner visits (both correct and incorrect) decreased, but learning performance was enhanced in young mice, while middle-aged mice were little affected by the introduction of air-puffs. Another, not mutually exclusive, explanation is an age-dependent change from behavioural flexibility towards perseverance. According to this view, young mice would show high levels of spontaneous alternation in corner visits, while middle-aged individuals may be more perseverant in their visit patterns. Under the low levels of reinforcement typical for the non-punished situation, young mice may remain more reactive to stochastic small distractions provoking curiosity, while middle-aged animals show more stable behaviour, resulting in better performance of a rapidly acquired place preference even after reversal of the position of the correct corner. However, after introduction of an aversive stimulus, young mice optimise their choice behaviour efficiently, while middle-aged mice continue to commit errors in visiting previously safe corners. In other words, even a relatively mild punishment, or stress,

challenges the ability of middle-aged mice to suppress previously acquired spatial preferences, despite their ability for learning spatial relations.

Nevertheless, one must also note that more young (5 out of 12) than middle-aged (0 out of 12) mice refused to enter the corners after having encountered air-puffs. These mice were removed from their respective IntelliCages due to having recorded zero licks during a 48-h period, thus indicating that they had not successfully learned to find the correct corner on repeated occasions. Thus, the results of the place- and reversal of place-learning with punishment apply to a (majority) sub-population of mice tolerating a generalised stress level. Mice refusing to perform certain tasks can also be found in a variety of conventional tests, but it is difficult to judge whether this results from randomly occurring environmental factors or whether the refusal represents a personality trait. In this study, however, the IntelliCage system easily recognised individual behavioural traits within inbred mice of the same strain, a finding of potential use for the study of epigenetic mechanisms.

An increase in the numbers of visits including licks was observed in young mice when punishment was introduced, resulting in similar behaviour in young and middle-aged mice. Thus, although young mice performed fewer corner visits under aversive stimulation, their motivation for visiting appeared to be aimed towards meeting physiological needs, rather than purely for exploration.

C57Bl/6J mice can be classified as mature adult (3–6 months), middle-aged (10–14 months) or old (18–24 months), corresponding to human age equivalents of 20–30 years, 38–47 years, and 56–69 years, respectively. Middle-aged mice are usually used in aging studies to determine whether certain age-related biomarkers change progressively or are first expressed in old age (Harrison, 2009). Observations in the wild have shown that only a minority of adult wild-living *Mus musculus* survives more than one winter in the northern hemisphere (Avenant and Smith, 2004), and no wild-living mice have reached the old age observed in the laboratory. Thus, in terms of natural biological aging, tissue wear and tear, and associated changes in the brain, it is logical to focus preferentially on comparing middle-aged mice with young adults. For example, one of the earliest “aging” effects observed in mice is a steep decline in adult hippocampal neurogenesis, the rate of newly generated neurones declining exponentially by 40% every month after 7 weeks of age and levelling off at very low rates at between 5 and 6 months (Ben Abdallah et al., in press). In this respect, the observation of impaired behavioural flexibility of middle-aged mice in spatial reversal learning under mild stress appears of interest, as it may relate to the low levels of adult neurogenesis for mice of this age.

A number of factors must be taken into consideration when analysing the results of the present study. Firstly, young and middle-aged C57Bl/6J mice did not originate from the same breeding colonies, due to the low availability of middle-aged and aged mice from commercial breeders. Therefore, subtle sub-strain differences unrelated to age cannot be ruled out. However, behaviourally relevant sub-strain differences have mainly been found in the genetically heterogeneous 129 lines (Montkowski et al., 1997), while they are rare in C57Bl/6 mice, the difference being associated with subtle co-variations in the size of the intra/infrapyramidal mossy fibre distribution and hippocampus-dependent behaviours, such as open-field exploration and radial maze learning (Jamot et al., 1994; Sluyter et al., 1999). Given the many strong differences observed in the IntelliCages, it seems unlikely that sub-strain differentiation played a major role in this study. Secondly, only female mice were used, due to the fact that they can be kept peacefully in cohorts over long periods, which is difficult and often impossible in males. Traditionally, males are preferred in behavioural studies using only one gender, apparently due to the concern that female

mice would show more variable behaviour because of their oestrous cycle. With respect to behavioural phenotyping this concern is unsubstantiated, at least for the C57Bl/6 strain used here (Meziane et al., 2007). Thirdly, the middle-aged mice were 14 months of age at the time of testing, which represents a suitable age for studying age-related cognitive decline, as outlined above. Certainly, future studies could also be performed using older mice, although a balance must be struck between testing “old” mice which are still sufficiently active and mobile to perform well within the IntelliCage system and not too old as to not survive an eight- to ten- week study.

In summary, the IntelliCage system is a powerful and efficient tool with which to detect subtle and distinct age-related changes in mice. Our data illustrate that the IntelliCage system can detect cognitive differences between middle-aged and young wild-type mice and that, in the absence of negative reinforcement, the natural instinct for exploration is too strong to permit effective learning. The results of the current study provide a basis for assessing the effects of putative cognitive-improving compounds in both young and middle-aged mice. We also suggest that analysis of middle-aged mice in this system provides an important tool aimed at discovery of nutritional compounds prolonging cognitive capacities *in vivo*.

## Acknowledgments

We thank K. Safi, C. Schiess, N. Ben Abdallah, S. Krackow and H.-P. Lipp for providing middle-aged C57Bl/6J mice, designing the test modules and helpful discussion of the results. Furthermore, we thank S. Krackow and H.-P. Lipp for critical evaluation of the manuscript.

## References

- Avenant NL, Smith VR. Seasonal changes in age class structure and reproductive status of house mice on Marion Island (sub-Antarctic). *Polar Biol* 2004;27:99–111.
- Barrett RJ, Leith NJ, Ray OS. A behavioral and pharmacological analysis of variables mediating active avoidance behavior in rats. *J Comp Physiol Psychol* 1973;82:489–500.
- Baskerville KA, Kent C, Nicolle MM, Gallagher M, McKinney M. Aging causes partial loss of basal forebrain but no loss of pontine reticular cholinergic neurons. *Neuroreport* 2006;17:1819–23.
- Ben Abdallah NM, Slomianka L, Vyssotski AL, Lipp HP. Early age-related changes in adult hippocampal neurogenesis in C57 mice. *Neurobiol Aging*; in press.
- Brinks V, van der Mark M, de Kloet R, Oitzl M. Emotion and cognition in high and low stress sensitive mouse strains: a combined neuroendocrine and behavioral study in BALB/c and C57Bl/6J mice. *Front Behav Neurosci* 2007;1(8):1–12.
- Chesler EJ, Wilson SG, Lariviere WR, Rodriguez-Zas SL, Mogil JS. Influences of laboratory environment on behavior. *Nat Neurosci* 2002a;5:1101–2.
- Chesler EJ, Wilson SG, Lariviere WR, Rodriguez-Zas SL, Mogil JS. Identification and ranking of genetic and laboratory environment factors influencing a behavioral trait, thermal nociception, via computational analysis of a large data archive. *Neurosci Biobehav Rev* 2002b;26:907–23.
- Crabbe JC, Wahlsten D, Dudek BC. Genetics of mouse behavior: interactions with laboratory environment. *Science* 1999;284:1670–2.
- Crawley JN, Paylor R. A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. *Horm Behav* 1997;31:197–211.
- Forster MJ, Dubey A, Dawson KM, Stutts WA, Lal H, Sohal RS. Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. *Proc Natl Acad Sci USA* 1996;93:4765–9.
- Francia N, Cirulli F, Chiarotti F, Antonelli A, Aloe L, Alleva E. Spatial memory deficits in middle-aged mice correlate with lower exploratory activity and a subordinate status: role of hippocampal neurotrophins. *Eur J Neurosci* 2006;23:711–28.
- Fukui K, Omoi NO, Hayasaka T, Shinikai T, Suzuki S, Abe K, et al. Cognitive impairment of rats caused by oxidative stress and aging, and its prevention by vitamin E. *Ann NY Acad Sci* 2002;959:275–84.
- Galsworthy MJ, Amrein I, Kuptsov PA, Poletaeva II, Zinn P, Rau A, et al. A comparison of wild-caught wood mice and bank voles in the IntelliCage: assessing exploration, daily activity patterns and place learning paradigms. *Behav Brain Res* 2005;157:211–7.
- Harrison DE. Maximum lifespan as a biomarker of aging. JAX Laboratories (<http://research.jax.org/faculty/harrison/ger1vLifespan1.html>); 2009.
- Jamot K, Bertholet JY, Crusio WE. Neuroanatomical divergence between two sub-strains of C57Bl/6J inbred mice entails differential radial-maze learning. *Brain Res* 1994;644:352–6.

- 694 Knapka E, Walasek G, Nikolaev E, Neuhäusser-Wespy F, Lipp H-P, Kaczmarek L, et al.  
695 Differential involvement of the central amygdala in appetitive versus aversive  
696 learning. *Learn Mem* 2006;13:192–200. 725
- 697 Lewejohann L, Skryabin BV, Sachser N, Prehn C, Heiduschka P, Thanos S, et al. Role of a  
698 neuronal small non-messenger RNA: behavioural alterations in BC1 RNA-deleted  
699 mice. *Behav Brain Res* 2004;154:273–89. 726
- 700 Lipp H-P, Vyssotski AL, Ben Abdallah N, Galsworthy MJ, Dell’Omo G, Amrein I. Hip-  
701 pocampal lesions in mice: behavioral effects in the water maze, naturalistic  
702 environment and Intelligage. In: Proceedings of the FENS annual meeting; 2004. 727
- 703 Lipp H-P. High-throughput and automated behavioural screening of normal and  
704 genetically modified mice. In: Future Drug Discovery 2005. London: Touch Brief-  
705 ings; 2005. 728
- 706 Lipp H-P, Litvin O, Galsworthy M, Vyssotski AL, Vyssotski DL, Zinn P, et al. Automated  
707 behavioral analysis of mice using INTELLICAGE: inter-laboratory comparisons  
708 and validation with exploratory behavior and spatial learning. In: Noldus LPJJ,  
709 Grieco F, Loijens LWS, Zimmermann HP, editors. Proceedings of measuring behav-  
710 ior 2005 (Wageningen 30 August to 2 September 2005). Fifth international  
711 conference on methods and techniques in behavioral research. Wageningen,  
712 Netherlands: Noldus Information Technology; 2006. p. 66–69. 729
- 713 Lister RG. Ethologically-based animal models of anxiety disorders. *Pharmacol Ther*  
714 1990;46:321–40. 730
- 715 Meziane H, Ouagazzal AM, Aubert L, Wietrzycz M, Krezel W. Estrous cycle effects on  
716 behavior of C57BL/6J and BALB/cByJ female mice: implications for phenotyping  
717 strategies. *Genes Brain Behav* 2007;6:192–200. 731
- 718 Montkowski A, Poettig M, Mederer A, Holsboer F. Behavioural performance in three  
719 substrains of mouse strain 129. *Brain Res* 1997;762:12–8. 732
- 720 Onishchenko N, Tamm C, Vahter M, Hökfelt T, Johnson JA, Johnson DA, et al. Develop-  
721 mental exposure to methylmercury alters learning and induces depression-like  
722 behavior in male mice. *Toxicol Sci* 2007;97:428–37. 733
- 723 Roozendaal B. Stress and memory: opposing effects of glucocorticoids on memory  
724 consolidation and memory retrieval. *Neurobiol Learn Mem* 2002;78:578–95. 734
- Singh A, Naidu PS, Kulkarni SK. Reversal of aging and chronic ethanol-induced cog-  
nitive dysfunction by quercetin a bioflavonoid. *Free Radic Res* 2003;37:1245–52. 726
- Sluyter F, Marican CC, Crusio WE. Further phenotypical characterisation of two sub-  
strains of C57BL/6J inbred mice differing by a spontaneous single-gene mutation.  
*Behav Brain Res* 1999;98:39–43. 727
- Sumien N, Heinrich KR, Sohal RS, Forster MJ. Short-term vitamin E intake fails to  
improve cognitive or psychomotor performance of aged mice. *Free Radic Biol*  
*Med* 2004;36:1424–33. 728
- Tecott LH, Nestler EJ. Neurobehavioral assessment in the information age. *Nat Neu-  
rosci* 2004;7:462–6. 730
- Verbitsky M, Yonan AL, Malleret G, Kandel ER, Gilliam TC, Pavlidis P. Altered hip-  
pocampal transcript profile accompanies an age-related spatial memory deficit  
in mice. *Learn Mem* 2004;11:253–60. 731
- Viosca J, Schuhmacher AJ, Guerra C, Barco A. Germline expression of H-RasG12V  
causes neurological deficits associated to Costello syndrome. *Genes Brain Behav*;  
2009 [Epub ahead of print]. 732
- Vöikar V, Polus A, Vasar E, Rauvala H. Long-term individual housing in C57BL/6J  
and DBA/2 mice: assessment of behavioral consequences. *Genes Brain Behav*  
2005;4:240–52. 733
- Vyssotski AL, Dell’Omo G, Poletaeva II, Vyssotski DL, Minichiello L, Klein R, et al. Long-  
term monitoring of hippocampus-dependent behavior in naturalistic settings:  
mutant mice lacking neurotrophin receptor TrkB in the forebrain show spatial  
learning but impaired behavioral flexibility. *Hippocampus* 2002;12:27–38. 734
- Wahlsten D, Rustay NR, Metten P, Crabbe JC. In search of a better mouse test. *Trends*  
*Neurosci* 2003;26:32–6. 735
- Wolfer DP, Lipp HP. Dissecting the behaviour of transgenic mice: is it the mutation,  
the genetic background, or the environment? *Exp Physiol* 2000;85:627–34. 736
- Wolfer DP, Litvin O, Morf S, Nitsch RM, Lipp H-P, Wurbel H. Laboratory animal wel-  
fare: cage enrichment and mouse behaviour. *Nature* 2004;432:821–2. 737