

## Behavioral and physiological effects of biotechnology procedures used for gene targeting in mice

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### Abstract

The effects of gene-targeting procedures on the behavior and physiological development of (chimeric) mice have been investigated. We used six groups of mice, each of them undergoing specific aspects of the biotechnological procedure, including electroporation, microinjection, and/or blastocyst culture. Changes in behavior and physiological development of the progeny (age 4–30 weeks) were investigated. Besides increased body weights, no significant difference between the six treatment groups and untreated C57BL/6 controls could be attributed to the biotechnology procedures. Therefore, we conclude that these procedures per se do not induce significant discomfort for the offspring. Differences in behavior, observed for the two groups of chimeric mice [one derived from electroporated embryonic stem (ES) cells and the other from nonelectroporated ES cells] when compared to the other (nonchimeric) groups, are, at least partly, due to the genetic background of the 129/Ola strain from which the ES cells are derived rather than to the biotechnological manipulations of the ES cells and/or blastocysts. The occurrence of hermaphrodites (8%) and some other gross pathologies observed in both groups of chimeric animals seem to indicate that developmental problems may occur when cells from different origin are simultaneously contributing to the development of one individual. This implies that during the production of gene-targeted mice, health and welfare of chimeric animals must be carefully monitored. © 2001 Elsevier Science Inc. All rights reserved.

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### 1. Introduction

Targeted gene mutation technology has resulted in many new knockout or knockin mouse models. These mutant mice have been generated by utilizing embryonic stem (ES) cells in which a gene (or part of a gene) is introduced through homologous recombination. Knockout mice, in which the native gene is silenced, are mostly used to reveal the *in vivo* function(s) of this gene. The technique of gene targeting has been extensively described [1–4]. Briefly, ES cells are cultured under conditions that prevent their differentiation yet allowing to retain their potential to repopulate

the entire embryo. During the *in vitro* culture of these pluripotent ES cells, exchange of native DNA with its mutated DNA segment (introduced by, e.g., electroporation) and selection of the clones with the mutant DNA take place. The mutant ES cells are then injected into the blastocyst of a donor animal. These blastocysts are introduced into the uterus of a foster mother. Several of these blastocysts develop into genetically chimeric animals. The extent of chimerism can be visualized if the ES cells and host blastocyst are derived from mice with different coat colors. If the ES cells, containing the integrated construct, contribute to the germ line, then the chimera can pass the transgene to its progeny. The heterozygous animals can be mated to generate a homozygous founder for the production of a knockout or knockin transgenic line. Most of the present ES cell-derived lines are knockout models. In order to study the validity of these new models, many behavioral studies have

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Table 1  
Biotechnological procedures used for the different groups of mice

Group	ES cells	Blastocysts
C	–	–
B	–	Blastocyst culture, transfer to recipients
BSh	–	“Sham” microinjection with M2 medium
Ch+/Ch–	Electroporation APC2 construct	Microinjection ES cells into blastocysts
NCh+/NCh–	No electroporation, culture ES cells	Microinjection ES cells into blastocysts

Blastocysts (C57BL/6) of all groups (except controls C) were cultured and transferred to foster mothers after biotechnological procedures. Production of B and BSh mice involved no ES cells. Production of Ch+ and Ch– mice involved culturing of ES cells (E14, 129/Ola), electroporation with APC2 construct, and microinjection of ES cells in the blastocysts. Production of NCh+ and NCh– mice involved culturing of ES cells and microinjection of those ES cells into the blastocysts without electroporation with DNA construct (C=controls, B=blastocysts, BSh=sham microinjection of blastocysts, N=no electroporation, NCh+ and Ch+=chimeric, NCh– and Ch–=nonchimeric).

been performed, especially in the areas of neuroscience and psychopharmacology [5–8]. Most of the current knockout mice are developed using ES cells derived from 129/Ola or 129/Sv inbred strains and blastocysts from the C57BL/6 (B6) inbred strain [9].

During gene targeting, cells and embryos are subjected to various *in vitro* manipulations like electroporation, microinjection, *in vitro* blastocyst culture, and blastocyst transfer. Until now, the effects of these specific manipulations have not been subjects of study. In particular, the consequences for health and welfare of the resulting offspring are unknown. Therefore, in the present study, different groups of mice were generated, each of which underwent different aspects of the gene-targeting technique (see Table 1) in order to determine if the manipulations had any effects on the development or behavior of the progeny mice. Specific effects of electroporation, microinjection, and blastocyst culture on the development of (chimeric) mice could be studied this way. The study has been performed in parallel to a project in which the possible role of adenomatous polyposis coli (APC)2, a homologue of the APC tumour suppressor, in development and sporadic colorectal cancer is being studied [10]. The APC2 knockout construct has been introduced by electroporation into ES cells, and both the chimeric and nonchimeric mice were used for our study.

All mice of the different groups were tested from 4 to 30 weeks of age in order to establish different aspects of behavior, such as locomotor activity, anxiety, and exploration of an unfamiliar environment. Also, the morphological/physiological development and clinical appearance has been monitored up until the age of 30 weeks, after which postmortem examinations were performed.

## 2. Animals and methods

### 2.1. Animals

Seven groups of mice, all with a different gene-targeting background (see Table 1), were used to study specific aspects of gene targeting like electroporation, microinjection, and blastocyst culture on the development of (chimeric) mice.

The control animals used in this study were normal C57BL/6 mice not subjected to any gene-targeting procedure.

One aspect in gene targeting is the isolation and culturing of blastocysts. To study the sole effect of this aspect of manipulation on health and welfare, blastocysts were isolated from C57BL/6 females and, after culturing, transferred to foster mothers. The resulting animals are indicated as B mice. Normally, the cultured blastocysts are first microinjected with the manipulated ES cells. By microinjection of M2 medium in cultured (C57BL/6) blastocysts (sham microinjection), the effect of the microinjection step on the blastocysts could be studied (BSh mice).

A mutant gene was introduced into the 129/Ola-derived ES cell line by the electroporation step. Subsequently, positive targeted clones were microinjected into the blastocysts (C57BL/6). ES cells and blastocysts are from two different strains with a different coat color. The resulting chimeric mice (Ch+ mice), which have a mixed fur color because these consist of a combination of both C57BL/6 and 129/Ola cells, were used to study effects of the gene-targeting procedure in chimeric mice that are carrying the mutant gene. Nonchimeric mice (Ch– mice), which were also born after the procedures were performed for the production of the Ch+ mice, served as controls for these mice; the ES cells have not contributed to the phenotype. These animals are comparable to BSh mice. In addition, to study the sole effects of chimerism (without the effect of the mutated gene), NCh+ chimeric mice were used, where the ES cells were directly microinjected into the blastocysts without the electroporation step (no transfection of the mutant gene).

Nonchimeric mice (NCh– mice), which were born after the procedures were performed for the production of the NCh+ chimerics, served as controls; like in the Ch– mice, the ES cells have not contributed to the phenotype of this group. To summarize, the following groups of animals were used.

#### 2.1.1. C mice: control animals, no gene-targeting procedures

These were animals of the C57BL/6 JIcoU inbred strain not submitted to any gene-targeting procedure. Twenty-two mice (13 males and 9 females) were tested.

### 2.1.2. *B* mice: untreated blastocysts transferred to foster mothers

Blastocysts were isolated, cultured, and transferred to foster mothers without any further gene-targeting treatment. Seventeen progeny mice were available for testing (10 males and 7 females).

### 2.1.3. *BSh* mice: blastocysts with “sham” microinjection transferred to foster mothers

Blastocysts were isolated, cultured, “sham” microinjected with M2 medium, and transferred to foster mothers without any further gene-targeting treatment. Nineteen progeny mice (12 males and 7 females) were available for testing.

### 2.1.4. *Ch*<sup>+</sup> mice: chimeric animals after electroporation of ES cells with *APC2* knockout gene

The *APC2* mouse gene construct (kindly provided by Van Es et al. [10]) was introduced into the 129/Ola-derived ES cell line by electroporation (250 V, 500  $\mu$ m, 7.8 ms) using standard procedures [12]. Positive targeted clones were used for microinjection into the blastocysts. Chimeric mice (as identified by mixed coat color) were obtained by injecting 10–15 gene-targeted ES cells into C57BL/6 blastocysts. An average of 10–12 injected blastocysts were transferred into pseudopregnant females (recipient mice). Fourteen chimeric mice were tested (7 females, 6 males, 1 hermaphrodite).

### 2.1.5. *Ch*<sup>–</sup> mice: nonchimeric animals after electroporation of ES cells with *APC2* knockout gene

Nonchimeric animals after procedures were performed for the production of the *Ch*<sup>+</sup> chimeras, as identified by their black coat color. Nine mice were tested (6 males and 3 females).

### 2.1.6. *NCh*<sup>+</sup> mice: chimeric animals without electroporation of ES cells

ES cells were cultured and directly microinjected into the blastocysts without any electroporation step (no transfection of a knockout gene). Chimeric mice (as identified by mixed coat color) were obtained by injecting 10–15 ES cells into C57BL/6 blastocysts. An average of 10–12 injected blastocysts were transferred into pseudopregnant females (recipient mice). Fourteen mice were tested (7 males, 6 females, 1 hermaphrodite).

### 2.1.7. *NCh*<sup>–</sup> mice: nonchimeric animals without electroporation of ES cells

Nonchimeric animals after procedures were performed for the production of the *NCh*<sup>+</sup> chimerics (as identified by their black coat color). Fifteen mice were available for testing (six males, nine females).

The control mice and all blastocysts used were from the same (black) inbred strain (C57BL/6 JIcoU; Central Laboratory Animal Institute, CLAI, Utrecht University,

Utrecht, The Netherlands). The ES cells used (E14 [11] provided by A. Berns of the Netherlands Cancer Institute, Amsterdam) were derived from the 129/Ola (agouti) strain. All foster mothers were B6D2F<sub>1</sub>/CrIBR (Charles River, Sulzfeld, Germany). The gene-targeting techniques were performed by an experienced technician according to standard procedures [12–14]. All animals were tested during the same period of postweaning development (age 4–30 weeks).

## 2.2. Housing

After weaning, at the age of 3–4 weeks, animals of the different groups were maintained as siblings and separated according to sex. They were housed in groups of two to three animals in wire-topped elongated Macrolon type II cages (530 cm<sup>2</sup>; Tecniplast, Rome, Italy) with sawdust bedding (Pinewood 3/4; Woodyclean, BMI, Helmond, The Netherlands). Per cage, a tissue (Kleenex, Kimberly-Clark, Ede, The Netherlands) was added for nest building. The tissues were renewed weekly at cage cleaning. Animals were housed conventionally and maintained under standard conditions (12:12 h light/dark cycle with lights on from 06:00 to 18:00 h, room temperature 19–25°C, relative humidity 40–70%). Food pellets (RMH-TM 1110; Hope Farms, Woerden, The Netherlands) and tap water were available ad libitum.

## 2.3. Body weight/clinical examination

Each week, throughout the study, mice were weighed individually, clinically examined, and inspected for any malformations or special traits. Mean body weight and growth rates (weight gain per week) were analysed for all groups for the whole test period.

## 2.4. Behavioral tests

During the 6 months of study, animals were subjected individually to several behavioral tests from weaning onwards. The different groups were tested in a randomized manner between 15.00 and 17.00 h. The tests, which were used to compare the development of the different groups, have been previously described [15]. In short,

### 2.4.1. Hole board test

Exploratory behavior was studied in a square 16-hole ( $\emptyset$  3 cm) board task, measuring 37.5  $\times$  37.5  $\times$  3.5 cm, covered with a transparent perspex lid (40  $\times$  40  $\times$  20 cm) [16,17]. The test was performed twice (at the age of 12 and 14 weeks) to study habituation as well. The number of holes explored during 3 min of testing was counted. A dip was recorded if a mouse dipped its head in a hole at least up to the eyes. Repeated dips into the same hole were not counted unless these were separated by locomotion. During testing, frequency of rearing at the walls of the lid, groom-

ing, and faeces and urine production were also registered for each mouse.

#### 2.4.2. Cage emergence test

A mouse is placed in an unfamiliar cage (Macrolon type I cage, size  $24 \times 13.5 \times 13$  cm, with a  $\emptyset$  4 cm hole in one sidewall, no lid on top), with its head opposite of the opening [17]. Its reactivity to escape (latency in seconds) from this novel environment (all four feet outside the cage) is measured at the age of 16 weeks. During testing, frequency of rearing at the walls of the cage, sniffing at the hole, freezing, grooming, and faeces and urine production were also recorded.

#### 2.4.3. Behavioral profile as registered by Laboratory Animal Behavior Observation, Registration and Analysis System (LABORAS)

The automated behavior registration system LABORAS (Metris, Hoofddorp, The Netherlands), validated by Van de Weerd et al. [18], has been described in detail [19,20]. With this system, the positions and six behavioral categories, namely immobility, locomotion, grooming, climbing, eating, and drinking, can be determined based on vibration patterns evoked by individual mice. Signals not recognised by LABORAS are classified as “undefined” (<10% of total). Introduction of a mouse in the LABORAS system always took place between 16:00 and 17:00 h, just prior to the dark period. Consequently, exploration, as induced by the unfamiliar housing situation, coincided with the normal activity pattern of the species. Mice were tested at the age of 22–24 weeks.

During 24 h, the behavior of a mouse was recorded to study the effect of the different gene-targeting procedures on circadian rhythms and time budgets of the animals. For analyses, the 24 h of the experiment were subdivided into eight time periods (1–3, 4–6, 7–9, 10–12, 13–15, 16–18, 19–21, and 22–24 h) after the start of the experiment. Every period, the relative mean time spent on each behavioral category was calculated and analysed.

Directly after the 24 h test, a metal climbing grid (size  $16 \times 10$  cm, mesh size  $0.5 \times 0.5$  cm) was placed into the cage (vertically attached to the cage lid) during the following 12 h dark period to study possible differences in climbing behavior. The relative mean time spent on climbing behavior with this extra climbing object was compared with the first four time periods of climbing without the object in the cages during the 24 h behavior test for the same animals.

#### 2.4.4. Light–dark test

Anxiety-related behavior was investigated in a light–dark test [21] by using a cage adapted [22] for LABORAS (Macrolon type III,  $38 \times 22 \times 27$  cm, two equally sized compartments, one illuminated by 1000 lx). A clear perspex tunnel ( $10 \times 6 \times 5$  cm) connected both compartments. Mice were placed in the dark compartment of the cage. For the next 10 min, LABORAS recorded the position of the animals (age of 20 weeks).

#### 2.4.5. Response to handling (during and after handling)

This test consisted of a manipulative phase during which the animal was subjected to different stimuli followed by an undisturbed observation of 10 min in their home cage. Testing was performed at the age of 28–30 weeks (animals have the same “handling” history) between 16:00 and 17:00 h. The behavioral response during marking of the tail was scored (ranging from 1 to 7; [23]), as well as several other responses of the animals (biting, freezing, or urine/faeces production) during the manipulation. Subsequently, the behavior of the animals after handling was observed in their home cage for 10 min using a sampling method, wherein every 5 s the behavior of the animal was scored according to a predefined ethogram based upon Blom et al. [24]. The following behaviors are distinguished: immobility, locomotion, rearing, grooming, digging, climbing, eating, social behavior, and fighting.

#### 2.5. Postmortem examinations

At the end of this study, animals were killed and postmortem macroscopic inspection was carried out on six males and six females (randomly chosen) of each group (except for Ch– mice, where all three females were examined). Subsequently, the heart, kidney, spleen, and liver were removed, blotted dry, and weighed. Whenever macroscopic abnormalities were detected, further microscopic examination was carried out.

### 3. Statistics

All data were statistically analysed using SPSS 9.0 for Windows to determine significant differences between the treatment groups and the control group. Where appropriate, variables were transformed logarithmically to promote homogeneity of variances and normality of the data. The body weight results (mean body weight and growth rate) were analysed by repeated-measurements analysis of variance (ANOVA). For the behavioral measurements, repeated-measurements ANOVA was also used for the hole board test, the (LABORAS) 24 h behavior test, and the LABORAS 12 h extra climbing test. A two-way ANOVA was used for the organ weights. The results of the cage emergence test and the light–dark test were analysed by one-way ANOVA. If ANOVA showed significant effects with respect to the behavioral measurements for groups and/or sex, treatment groups were compared to the control group (C) by using Dunnett post hoc tests; multiple comparisons between treatment groups were Bonferroni corrected. Behavior performed by mice during the hole board test, the cage emergence test, and the handling test was analysed using nonparametric statistics, i.e., the Kruskal–Wallis test followed, where appropriate, by nonparametric multiple comparisons tests [25]. The level of statistical significance was preset at  $P < .05$  for all parameters. All data are presented as mean

values  $\pm$  S.E.M. If sex differences were not statistically significant, data from male and female mice were pooled.

Data of the two hermaphrodites in Ch<sup>+</sup> and NCh<sup>+</sup> groups were excluded in case of statistically significant differences between the sexes.

## 4. Results

### 4.1. Survival rate

No major effects of the different gene-targeting treatments were found on survival rates. Only one B female died with unknown cause at week 6.

### 4.2. Body weight/growth rate

Fig. 1 shows the average body weights of the mice during the experiment. Repeated-measures ANOVA

revealed significant effects for group [ $F(6,94)=14.78$ ,  $P<.001$ ] and gender [ $F(1,94)=93.30$ ,  $P<.001$ ] for the body weights. Both male and female mice from all treatment groups weighed more than the control C mice (C57BL/6) for the whole postweaning period ( $P<.001$ ), but no differences in growth rate (weight gain/week) were detected. The heaviest mice were the chimeric NCh<sup>+</sup> males and Ch<sup>+</sup> females. Multiple comparisons demonstrated no significant differences in body weight between the other groups. Overall, males weighed significantly more than females ( $P<.001$ ) for all groups.

### 4.3. Exploratory behavior

Repeated-measures ANOVA revealed a significant effect of group on the number of holes explored in 3 min [ $F(6,94)=9.86$ ,  $P<.001$ ], while no effects for gender were detected. Multiple comparisons demonstrated that chimeric Ch<sup>+</sup> mice expressed significantly ( $P<.01$ ; Fig. 2) less

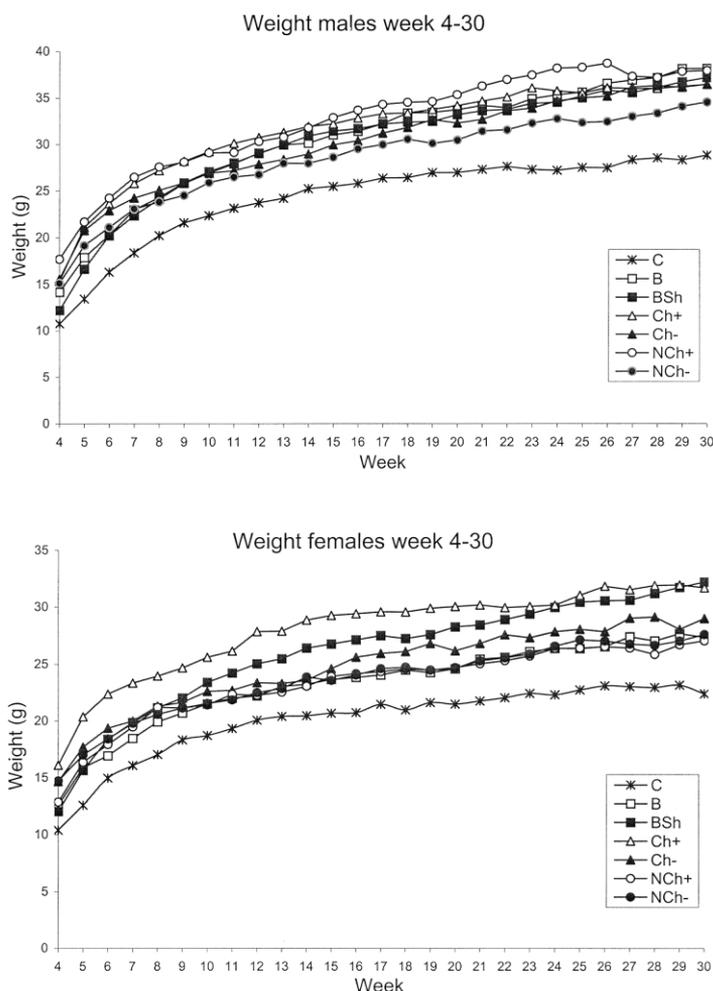


Fig. 1. Mean male (a) and female (b) body weight (g) in postweaning period (age 4–30 weeks). C: control animals (no gene-targeting techniques; B6); B: blastocysts untreated transferred to foster mothers (B6); BSh: blastocysts with “sham” microinjection transferred to foster mothers (B6); Ch<sup>+</sup>: chimeric animals after electroporation with APC2 knockout gene (B6/129); Ch<sup>-</sup>: nonchimeric animals after electroporation with APC2 knockout gene (B6); NCh<sup>+</sup>: chimeric animals without electroporation (B6/129); NCh<sup>-</sup>: nonchimeric animals without electroporation (B6).

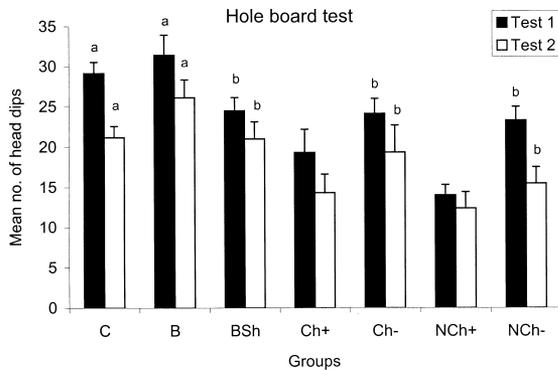


Fig. 2. Exploratory behavior in two subsequent hole board tests. Testing was performed at the age of 12 (Test 1) and 14 weeks (Test 2) between 15:00 and 17:00 h. Test period was 3 min. Data are expressed as mean  $\pm$  S.E.M. numbers of head dips. The chimeric Ch+ and NCh+ mice were less explorative compared to the other groups in both tests. (<sup>a</sup>  $P < .01$ , significant difference compared to both Ch+ and NCh+ groups; <sup>b</sup>  $P < .05$ , significant difference compared to NCh+ group only).

exploratory activity than control (C) mice and B mice in both tests. In addition, the chimeric NCh+ mice displayed significantly ( $P < .05$ ) less exploratory activity than all other groups except Ch+ in both hole board tests. All groups showed a significant decrease in number of holes explored in Test 2 compared to Test 1 [ $F(1,94) = 24.01$ ,  $P < .001$ , mean 23.77 vs. 18.84]. During the first hole board test, BSh, Ch+, and NCh+ mice showed significantly less rearing to the walls of the transparent lid compared to the controls ( $P < .01$ ), while the faeces production of Ch+ and NCh+ mice was significantly higher than the control group ( $P < .01$ ). Overall, males produced significantly more faeces and urine and performed more grooming behavior than females for all the groups ( $P < .01$ ).

#### 4.4. Cage emergence test

There was a significant effect of group on the time to escape from an empty cage [ $F(6,101) = 3.58$ ,  $P < .01$ ]. Multiple comparisons demonstrated that chimeric Ch+ mice needed significantly more time to escape (Fig. 3) compared to the control C mice ( $P < .01$ ), B mice ( $P < .05$ ), and NCh- mice ( $P < .05$ ). All animals left the cage within the maximum time set for the test (10 min). Most animals left the cage within 60 s. However, Ch+, NCh+, and BSh mice showed greater variation in time to escape when compared to the other groups ( $P < .05$ ). No differences between males and females were found between groups. Also, no significant differences between groups were found for the various behaviors scored during the test. The behaviors most frequently observed were rearing to the sidewalls and sniffing at the hole.

#### 4.5. Light–dark test

In the light–dark test, the mean number of movements from the dark to the light compartment and vice versa

(crossings) as well as the latency to leave the dark compartment for the first time and the total times spent in the light or in the dark compartment were recorded (see Fig. 4a and b). Gender effects were only observed for the latency.

Overall, there was a significant effect of group for the number of crossings [ $F(6,91) = 4.21$ ,  $P < .01$ ]. Multiple comparisons demonstrated that chimeric Ch+ and NCh+ mice showed significantly less crossings (see Fig. 4a) compared to the B mice ( $P < .05$ ). The Ch+ mice also showed fewer crossings compared to the NCh- animals ( $P < .05$ ). In addition, the B mice displayed significantly more crossings than the controls ( $P < .05$ ). For the other groups, no significant differences could be demonstrated in number of crossings.

No significant differences were found for latency until the first entry in the other compartment between the groups (Fig. 4b). However, both chimeric Ch+ and NCh+ mice showed greater variation in time until their first entry compared to the other groups ( $P < .01$ ). For all groups, effects in males and females were similar, although overall males showed increased latency compared to females [ $F(2,91) = 3.71$ ,  $P < .05$ ; overall mean males 131.4 s vs. mean females 96.3 s].

Mice from all groups showed a preference for the dark compartment, as measured by total time spent in the dark vs. light during the 10 min test sessions. Overall, there was a significant effect of group for time in the light compartment [ $F(6,91) = 4.29$ ,  $P < .01$ ]. Total time spent in the light is significantly shorter for Ch+ and NCh+ mice compared to the control group ( $P < .05$ ) and the nonchimeric Ch- and NCh- groups ( $P < .01$ ).

#### 4.6. LABORAS 24 h test

Fig. 5 presents the results of the 24 h behavior observation as recorded by LABORAS. Per time period, the relative mean time spent on each of the six different behavior categories is shown for each of the groups. The category “undefined,” which is on average less than 10% of the total

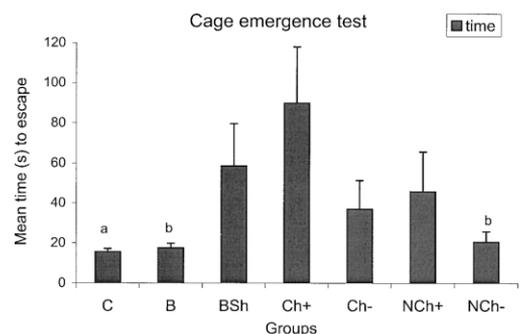


Fig. 3. Mean  $\pm$  S.E.M. time (s) to escape from an empty cage. Testing was performed at the age of 16 weeks between 15:00 and 17:00 h. Maximum testing period was 10 min. Differences in reactivity were found for chimeric Ch+ mice compared to some other groups. (<sup>a</sup>  $P < .01$ , <sup>b</sup>  $P < .05$ , significant difference compared to Ch+ group).

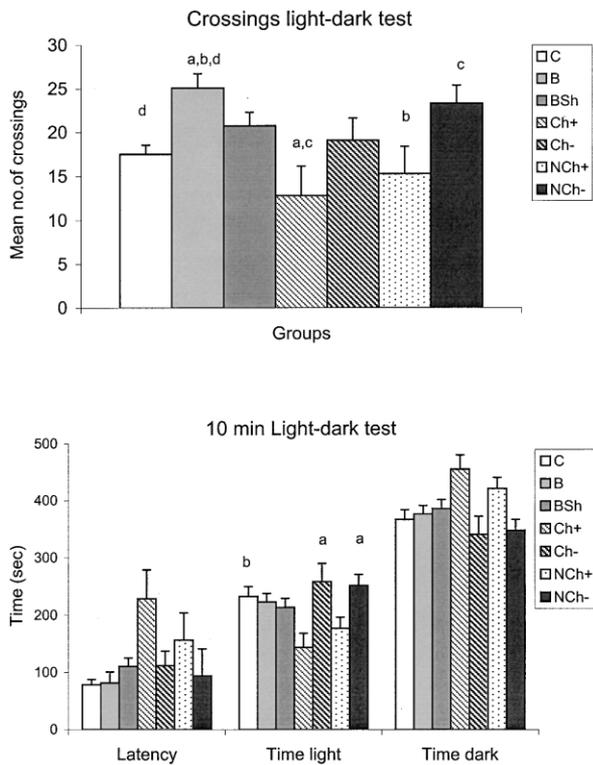


Fig. 4. Results of the light–dark preference test. (a) Mean number of crossings between the two compartments; (b) light–dark test, start from dark compartment. Number of crossings between the two compartments (a), latency to first entry (b), and total time spent in the light (b) and in the dark (b) compartments (in s) of the light–dark test (mean  $\pm$  S.E.M.) are shown. Animals of each group were placed in the dark compartment at the start of the experiment. Testing was performed at the age of 20 weeks between 15:00 and 17:00 h. Test period was 10 min. The chimeric Ch+ and NCh+ mice were more anxious compared to the other groups. (a) Bars with equal superscripts are significantly different,  $P < .05$ ; (b) <sup>a</sup>  $P < .01$ , <sup>b</sup>  $P < .05$ , significant difference compared to both Ch+ and NCh+ groups.

time, is not shown. Lights went out in the 1–3 h period and went on again in the 13–15 h period. Gender effects were present for climbing and eating behavior.

#### 4.7. Immobility (Fig. 5a)

Overall, there was a significant group effect for immobility [ $F(6,90) = 2.91$ ,  $P < .05$ ]. During the 1–3 h, 4–6 h, and 7–9 h periods, Ch+ mice showed more immobility compared to the controls ( $P < .01$ ) and all other groups ( $P < .05$ ), except the NCh+ mice. Although the NCh+ mice also showed a similar pattern, their immobility was significantly increased compared to all other groups for the 4–6 h period only ( $P < .05$ ). During the light period, no significant differences in duration of immobility were found between the groups.

#### 4.8. Locomotion (Fig. 5b)

Overall, ANOVA revealed a significant group effect for locomotion [ $F(6,90) = 4.86$ ,  $P < .001$ ]. The Ch+ mice

showed significantly less locomotion compared to control group C ( $P < .01$ ) and all other groups ( $P < .05$ ), except NCh+ mice, mainly during the dark period (1–3 h, 4–6 h, and 7–9 h periods). The NCh+ mice also showed less locomotion though not statistically significant different from the other groups ( $P < .1$ ).

#### 4.9. Climbing (Fig. 5c)

Overall, the chimeric Ch+ and NCh+ mice spent significantly less time on climbing compared to the nonchimeric NCh– mice [ANOVA group effect:  $F(6,90) = 4.98$ ,  $P < .001$ ; 1–24 h, Ch+ and NCh+ vs. NCh–:  $P < .01$ ]. These differences were mainly caused by their climbing behavior during the first three periods (dark period). No significant differences between the other groups were found. Overall, females spent more time on climbing than males for all groups [ $F(1,90) = 16.22$ ,  $P < .001$ ; mean 10.36% vs. 6.64%].

#### 4.10. Grooming (Fig. 5d)

No significant differences were found in grooming behavior for all groups. Overall, the mean percentage of time spent on grooming was 13%.

#### 4.11. Drinking (Fig. 5e)

For the 1–3 h, 4–6 h, and 7–9 h periods, the B and BSh– mice showed significantly less drinking behavior compared to the controls [ANOVA group effect:  $F(6,90) = 2.25$ ,  $P < .05$ ; B and BSh– vs. controls:  $P < .05$ ].

#### 4.12. Eating (Fig. 5f)

The Ch+ and NCh+ mice showed significantly reduced eating behavior when compared to the controls during the first two periods [ANOVA group effect:  $F(6,90) = 2.79$ ,  $P < .05$ ; Ch+ and NCh+ vs. controls:  $P < .05$ ]. For all groups, effects for males and females were similar, although overall females showed more eating behavior compared to males [ $F(1,90) = 7.24$ ,  $P < .01$ , overall mean females 5.2% vs. mean males 4.1%].

#### 4.13. LABORAS 12 h extra climbing

Directly after the 24 h test, animals were tested for extra climbing behavior by adding an extra climbing grid to the cage. During the following 12 h (dark period), the climbing behavior was recorded. Overall, females spent more time on climbing than males for all groups [ $F(6,31) = 2.52$ ;  $P < .05$ ]. This difference is less significant for the Ch+ and NCh+ chimeric mice, where both males and females showed less climbing behavior (data not shown). Overall, mice of all groups did not spend significantly more time on climbing when the extra climbing grid was added to their cage.

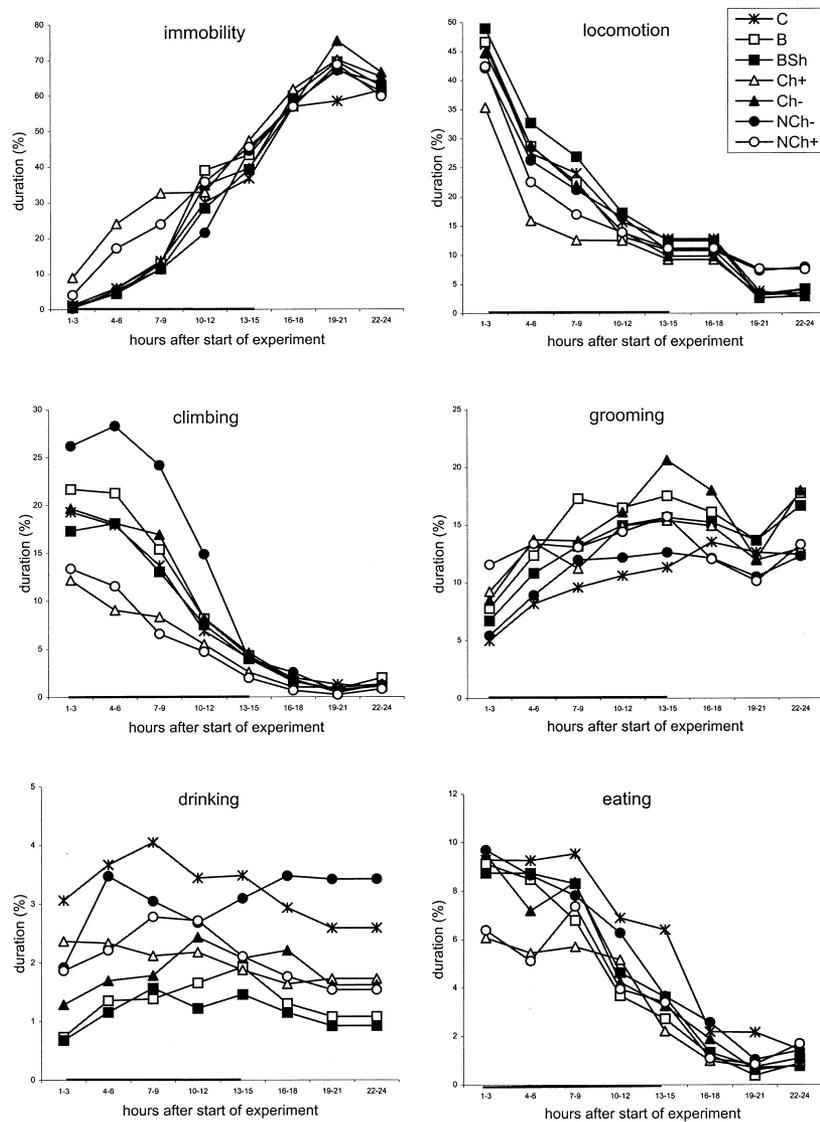


Fig. 5. Results of the LABORAS behavior registration system, 24 h test. Per time period of 3 h, the relative mean time spent on each of the six behavioral categories is shown for mice of all groups during 24 h of testing. The data of the category “undefined” are not shown. Lights went out in the 1–3 h period and on again in the 13–15 h period (black bars indicate dark period). Mice were tested at the age of 22–24 weeks. In the dark period, chimeric Ch+ and NCh+ mice spent less time on locomotion and climbing and more time on immobility than the other groups.

#### 4.14. Handling test

The score of the response to handling and the behavior after handling scored for 10 min in the animal’s home cage did not reveal any overall significant difference between the groups (results not shown).

#### 4.15. Postmortem examinations

Significant differences were found for the adult body weight between the males and females of the treatment groups and the control group at the postmortem examination [ $F_{\text{males}}(6,34) = 3.64, P < .01$ ;  $F_{\text{females}}(6,39) = 6.26, P < .001$ ; Table 2, see also Fig. 1). Males of all the treatment groups and BSh, Ch+, and NCh – females were all heavier than the

controls ( $P < .05$ ). Due to these differences in total body weight, statistical analysis was performed on both the absolute and the relative organ weights (g/total body weight). For the absolute and relative weight of the spleen and heart, no significant differences were found between groups and gender. Absolute kidney weights were higher for Ch+ and NCh+ males [ANOVA group effect:  $F(6,34) = 3.01, P < .01$ ; Ch+ and NCh+ vs. controls:  $P < .01$ ] and Ch+ and BSh females [ANOVA group effect:  $F(6,39) = 3.83, P < .01$ ; Ch+ and BSh vs. controls:  $P < .05$ ] compared to the control mice. After analysis of the absolute liver weight, significant differences were detected for B and Ch – males compared to the control group [ANOVA group effect:  $F(6,34) = 3.51, P < .01$ ; B and Ch – vs. controls:  $P < .05$ ], while this was also apparent for the BSh, Ch+, and NCh – females [ANOVA group effect:

Table 2  
Absolute organ weights as mean  $\pm$  S.E.M. grams  $\times 10^{-2}$ , split by group and gender

Organ	C	B	BSh	Ch+	Ch-	NCh+	NCh-
Bodyweight							
Males (g)	30.96 $\pm$ 1.01	41.03 $\pm$ 1.80 <sup>a</sup>	37.12 $\pm$ 0.53 <sup>a</sup>	37.41 $\pm$ 1.51 <sup>a</sup>	38.74 $\pm$ 2.26 <sup>a</sup>	39.39 $\pm$ 2.33 <sup>a</sup>	37.22 $\pm$ 1.76 <sup>a</sup>
Females (g)	23.47 $\pm$ 0.34	27.99 $\pm$ 0.81	32.60 $\pm$ 1.69 <sup>a</sup>	32.94 $\pm$ 1.87 <sup>a</sup>	29.02 $\pm$ 1.90	25.42 $\pm$ 1.45	29.87 $\pm$ 1.44 <sup>a</sup>
Spleen							
Males	7.16 $\pm$ 0.31	8.33 $\pm$ 0.49	9.17 $\pm$ 1.01	8.00 $\pm$ 0.45	7.67 $\pm$ 0.56	8.80 $\pm$ 0.58	7.50 $\pm$ 0.34
Females	8.50 $\pm$ 0.43	9.00 $\pm$ 0.58	10.67 $\pm$ 0.71	11.43 $\pm$ 0.99	8.67 $\pm$ 0.88	10.7 $\pm$ 2.64	10.17 $\pm$ 0.70
Heart							
Males	15.17 $\pm$ 0.70	17.67 $\pm$ 0.56	18.67 $\pm$ 1.67	19.00 $\pm$ 0.82	15.83 $\pm$ 0.31	18.20 $\pm$ 1.11	16.83 $\pm$ 0.98
Females	11.50 $\pm$ 0.22	12.00 $\pm$ 0.68	16.50 $\pm$ 2.39	16.57 $\pm$ 1.44	12.00 $\pm$ 1.53	20.67 $\pm$ 7.33	13.50 $\pm$ 0.85
Kidney							
Males	20.00 $\pm$ 1.09	24.17 $\pm$ 0.91	23.17 $\pm$ 1.13	25.83 $\pm$ 1.38 <sup>b</sup>	23.17 $\pm$ 0.79	26.20 $\pm$ 1.28 <sup>b</sup>	22.83 $\pm$ 1.56
Females	14.33 $\pm$ 0.82	17.00 $\pm$ 0.68	19.33 $\pm$ 1.89 <sup>a</sup>	21.57 $\pm$ 1.43 <sup>a</sup>	17.67 $\pm$ 0.88	17.00 $\pm$ 0.63	18.00 $\pm$ 1.00
Liver							
Males	148 $\pm$ 6.28	226 $\pm$ 23.4 <sup>a</sup>	169 $\pm$ 8.07	173 $\pm$ 9.59	199 $\pm$ 11.4 <sup>a</sup>	181 $\pm$ 19.6	177 $\pm$ 7.22
Females	107 $\pm$ 2.14	135 $\pm$ 5.73	152 $\pm$ 5.79 <sup>a</sup>	169 $\pm$ 10.7 <sup>a</sup>	130 $\pm$ 9.39	114 $\pm$ 4.36	141 $\pm$ 11.2 <sup>a</sup>

Values are means of six males or six females per group ( $\pm$  S.E.M., in grams  $\times 10^{-2}$ ), except for Ch- mice (three females). Body weights are shown in grams.

<sup>a</sup>  $P < .05$ , significant difference compared to control C mice.

<sup>b</sup>  $P < .01$ , significant difference compared to control C mice.

$F(6,39) = 8.15$ ,  $P < .001$ ; BSh, Ch+ and NCh- vs. controls:  $P < .05$ ]. For the relative weight of the kidney and liver, no significant differences were found between groups and gender, except between liver weights of B and BSh- males [ANOVA group effect:  $F(6,34) = 3.39$ ,  $P < .05$ ; B vs. BSh- :  $P < .05$ ] due to some extreme fat livers of B males.

Postmortem evaluation revealed some obvious differences between the various groups, mainly for the Ch+ and NCh+ chimeric mice. In both chimeric groups, one presumed male turned out to be hermaphrodite, visible by the presence of ovary ( $4 \times 2$  mm), oviduct, and uterus on one side and small testicle ( $3 \times 2 \times 2$  mm) and spermatiduct on the other side. The presence of follicles at different stages and corpora lutea suggested that the ovaries were active, while the testicles showed spermatogenesis with spermatocytes and spermatids but no spermatozoa. In the epididymis, no viable spermatozoa were present. Furthermore, one chimeric Ch+ male had a small opening in the diaphragm and one Ch+ female showed severe hydronephrosis (almost complete atrophy of the kidney). Of the chimeric NCh+ group, two females showed heart lesions. One mouse had a very large heart (570 mg vs. mean 130 mg), due to intimal proliferation and stenosis of the first part of the aorta, and chronic congestion of the lungs with many iron-loaded macrophages and some petechiae. The other one had severe round cell myocarditis in both atria. In both chimeric groups, signs of focal round cell pyelonephritis (one Ch+ female, three NCh+ females, and one hermaphrodite) and focal round cell pyelitis (one Ch+ female, one NCh+ female, and one hermaphrodite) were detected.

## 5. Discussion

The present study was performed to investigate the impact of the biotechnology procedures, involved in gene

targeting, on behavioral and physiological parameters in mice during their postweaning development.

At weaning, the average body weight of all treatment groups was higher than the control C57BL/6 mice. During the whole postweaning period, all mice of the treatment groups were heavier than the controls, but they did not show a higher growth rate. The fact that gene-targeting techniques affect body weight has also been reported for other mammalian species, including cattle and sheep [26], where transfer of bovine and ovine embryos, produced by in vitro procedures or by nuclear transfer, has resulted in the birth of offspring with increased body weight. An explanation could be that when using gene-targeting techniques, there is a selection for superior material (the best-looking blastocysts are reimplanted), subsequently causing the higher body weights. Another explanation may be that the uterine environment of the foster mothers provides better conditions for the embryos than the uterine environment of the same inbred strain. It is unlikely that this difference in body weight is a 129 strain effect. The higher body weights were not only found in the chimeric groups (Ch+ and NCh+) but also in the nonchimeric groups (B; BSh; Ch- and NCh-).

By using behavioral tests, we could not find such an overall difference between treatment groups and control animals. However, we have shown that the chimeric mice (groups Ch+ and NCh+) differ substantially in their behavior when compared to the control animals or to the other treatment groups.

When comparing the results of both hole board tests, the Ch+ and NCh+ mice appeared hypoactive compared to the controls and B mice, while NCh+ mice were also hypoactive compared to the other groups. During the first test, they also showed less rearing to the sidewalls and produced more faeces than control mice, all together indicating a higher state of anxiety. All groups showed a decrease in dips

in the second hole board test, which is in line with previous results [15]. The animals were less active and more hesitant, sniffed more, and walked less deliberately, which might indicate that explorative behavior is diminished due to habituation with time or reduced curiosity [17,27]. Apparently, there are no differences in such habituation between the various treatment groups.

Chimeric mice also differed in the cage emergence test, but this reached significance for the Ch<sup>+</sup> mice only. The chimeric NCh<sup>+</sup> mice showed a greater variation in time to escape from the novel environment. Although all animals escaped from the cage within 10 min, and the majority even within 60 s, more Ch<sup>+</sup> and NCh<sup>+</sup> mice escaped after these 60 s than in the other groups. This could indicate enhanced anxiety [21].

The light–dark test has frequently been used to test anxiolytic properties of new drugs [28]. The choice to move from dark to light confronts the animal with a conflict between the drive to explore the new environment and the aversion for bright light. In the present study, mice of all groups spent less time in the light compartment, having a preference for the dark enclosed space. The chimeric mice exhibited a higher level of anxiety than animals of the other groups when confronted with the constraining light–dark choice test, as shown by greater variation in latency, less number of crossings, and less time spent in the light compartment.

The results of the 24 h behavior observations showed similar behavioral circadian patterns for all groups, except for the two groups of chimeric mice. High levels of activity associated with exploration were observed in the first 3 h (cf. locomotion and climbing). The animals continued to be active during the dark period. When the lights turned on again (13–15 h period), resting increased. Grooming was fairly constant during the whole 24 h period. These behavioral patterns are consistent with circadian rhythms of mice as found by others [18,20]. The chimeric Ch<sup>+</sup> and NCh<sup>+</sup> mice showed a similar pattern but spent less time on locomotion and climbing and more time on immobility than the other groups. This has also been found for 129 mice used in a similar test situation [29]. Adding an extra climbing object to the LABORAS cage as environmental enrichment did not result in increased climbing behavior in any of the groups.

No significant differences in responsiveness to handling were found. This might be due to the fact that all animals were frequently handled from birth and thus were used to the handling routines. Also, the chimeric Ch<sup>+</sup> and NCh<sup>+</sup> mice showed no increased (stress) response to handling.

The fact that no differences in behavior between the control group and the four groups of nonchimeric animals were found indicates that the biotechnological procedures have no impact on the behaviors as tested in this study. Therefore, the behavioral differences as found between chimeric and nonchimeric mice can most likely be ascribed to the chimerism *per se* rather than to the biotechnological

procedures. The ES cells used in this study were derived from the 129/Ola strain and introduced in C57BL/6 (B6) blastocysts. Consequently, the chimeric mice contained cells of both strains and therefore can display behavior of both strains [9,30,31]. Previous studies have shown that the two inbred strains display marked differences in performance in a variety of behavioral tasks [32–34]: the B6 strain is more explorative and less anxious than the 129 strain. Therefore, the observed differences in behavior between the chimeric and nonchimeric animals can not be interpreted in terms of impaired welfare but are most likely due to the contribution of the 129/Ola strain to the chimeric animals.

By postmortem examinations, in both chimeric groups, one hermaphrodite was discovered. When the recipient blastocyst is of a different sex genotype than the ES cells, the resulting embryo is a sex chimera with both XX and XY cells. Such a sex chimera may develop into a phenotypic male, female, or hermaphroditic mouse, depending on the genotype of the embryonic cell that developed into sex-determining structures [35]. Although the occurrence of hermaphroditism in chimeric animals has previously been reported [35,36], chimeric mice that are used for normal breeding are usually not clinically examined, and thus, the presence of hermaphrodites is not likely to be detected. It seems of interest to screen chimeric animals in this respect, because this might be one of the major causes of the fertility problems encountered when breeding chimeras.

Several other pathologies were found during gross post-mortem examinations, mainly with chimeric mice. Microscopic evaluation revealed even more lesions, such as pyelitis, pyelonephritis, myocarditis, and intimal proliferation near the aortic valves. However, microscopy of non-chimeric mice was not performed, since no macroscopic lesions were detected. These findings indicate that, when chimeric animals are produced, careful postmortem examinations may reveal that the welfare of these animals is compromised more severely than can be concluded from the behavioral and physiological screenings as performed in the present study. No signs of colon cancer were present in the chimeras with the APC2 knockout construct (Van Es, personal communication). It seems that the construct was not effective as far as colon cancer is concerned.

## 6. Conclusion

Besides a higher body weight, no significant effects of manipulating ES cells and blastocysts were detected. Neither the *in vitro* culturing and the “sham” microinjection of blastocysts (B and BSh vs. C mice) nor the microinjection of ES cells into blastocysts (NCh – vs. C; B and BSh mice) or the electroporation of the ES cells (Ch – vs. C; B; BSh and NCh – mice) seem to have a major effect on the normal development.

The results of this study indicate that behavioral differences were most apparent in chimeric (Ch<sup>+</sup> and NCh<sup>+</sup>)

mice. These animals were less explorative, more anxious, and seem to habituate slower in novel situations than the other mice. These are characteristics of the 129 rather than of the C57BL/6 strain. Based on the results of the behavioral tests, it might be concluded that chimerism does not substantially decrease the welfare of these animals. However, since postmortem examinations revealed a high percentage (8%) of hermaphrodites and several other pathological lesions among the chimeras, this conclusion needs some caution. It is recommended that in future gene-targeting experiments, postmortem examinations of both chimeric and nonchimeric animals is applied on a routine basis in order to further evaluate the consequences of chimerism for the well-being of the animals.

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