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Impact of transgenic procedures on behavioral and physiological responses in postweaning mice

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Abstract

This study evaluates the effects of biotechnological procedures involved in the process of microinjection-induced transgenesis in the mouse by comparing four groups of C57BL/6 mice that differ in their transgenic background (transgenics after integration of a functional corticotropin-releasing factor (CRF) gene construct, transgenics after integration of a nonfunctional CRF gene construct, nontransgenics after transgenic procedures, and controls). These four groups have been tested in various behavioral paradigms. Moreover, the development in growth, morphological characteristics, and clinical appearance of the animals have been recorded from 4 till 30 weeks of age. Differences in behavior, weight gain, and morphology were found between Group 1 (transgenic CRF animals) and Group 4 (control animals). For Group 2 (animals with a noncoding construct) and Group 3 (nontransgenic animals after transgenic procedures), no significant differences from control animals were found. This indicates that, under the present conditions, the biotechnological procedures related to transgenesis (microinjection, in vitro culture, and embryo transfer) have no significant effect on the normal development of the mice in the postweaning period. These results substantiate previous findings on these animals, obtained by screening them in the preweaning period (Days 0–21). However, before general conclusions as to what extent the technique of transgenesis affects the welfare of the animals can be drawn, more and different transgenic lines should be studied in this or a similar way.

Keywords: Transgenic procedures; Transgenic mice; Behavioral tests; Welfare; Corticotropin; Microinjection

1. Introduction

Most transgenic animals are produced by microinjection or embryonic stem cell techniques. After microinjection, the random integration of the DNA into the genome may increase the chance of disturbing normal physiological processes, which may affect the welfare of the animal [1–3]. Not only the expression of the transgene, but also the manipulation of the oocytes or embryos or the disruption of parental DNA at the integration site of the gene construct (insertional mutations) can influence normal development [4].

We have started a study to identify and quantify physiological and behavioral differences in four groups of animals of the same mouse inbred strain differing only in their transgenic background (functional gene construct integrated, nonfunctional gene construct integrated, transgenic technique without integration, and no transgenic treatment, respectively). This approach aims to differentiate the effects of the biotechnological procedures per se from the effects caused by the expression of the transgene. The study has been performed parallel to a current project at Utrecht University, where the corticotropin-releasing factor (CRF) gene was introduced into the genome of C57BL/6 mice by microinjection. These transgenic mice are under investigation as a putative model of depression. C57BL/6 is an inbred strain widely used as a reference strain for the maintenance of numerous mutations, affecting, in particular, physiology and behavior [5]. It has become a reference strain for comparisons in various research fields such as, e.g., hematology and cancer chemotherapy and is now also commonly used in transgenic and gene-targeting research. Compared to other inbred strains, the C57BL/6 strain is more active and less anxious [6].

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The first part of the study emphasized the search for differences in the early postnatal development of the four experimental groups. To this end, newborn mice were subjected to various behavioral tests and the growth and development of their morphological characteristics were recorded from birth to 3 weeks of age. A test protocol was developed for routine observations of the animals and for collecting information that might be relevant for establishing the impact of transgenesis on the welfare of these animals [7]. The results of the preweaning study have indicated that the presence of the microinjected DNA construct (both functional and nonfunctional) influenced the survival rate during the first 2–3 days after birth. In both groups, the average loss of pups was about 10%, in contrast to the groups without the presence of the DNA construct, in which none of the pups died. During the first 11 days after birth, the increase in body weight was significantly lower for the pups with a functional CRF construct and higher for the nontransgenic pups after transgenic procedures, compared to the control group. No significant differences in behavior and/or morphological development were observed between the four groups in the preweaning period [7].

In the present study, the same groups of mice are monitored in their postweaning period, in order to compare different aspects of behavior such as locomotor activity, anxiety, and exploration of an unfamiliar environment. Also, their morphological/physiological development is monitored up until the age of 30 weeks, after which postmortem examinations were performed.

2. Animals and methods

2.1. Animals

Four groups of mice, all from the same inbred strain (C57BL/6N Crl; Broekman, Someren, The Netherlands) were used in this study. All animals were tested during the same period of postweaning development (age 4–30 weeks) and differed only in their transgenic backgrounds.

2.1.1. Group 1: CRF transgenic animals

This group consisted of two subgroups of 25 (Group 1a: 9 females and 16 males; 6 litters) and 23 (Group 1b: 13 females and 10 males; 6 litters) transgenic mice. Each subgroup was derived from a different CRF transgenic founder male (animal carrying the transgene), which was crossed with a wild-type C57BL/6N Crl female. The CRF construct consisted of the rat genomic CRF gene with the 5′ regulatory region of the mouse Thy-1 gene, which directs expression to the brain. This Thy-1-CRF fusion gene was microinjected into the male pronucleus of fertilized eggs of a C57BL/6N Crl female. Injected eggs were implanted into pseudopregnant foster mothers. To identify transgenic founder animals, tail DNA from offspring was screened by standard Southern dot–blot analyses. The offspring of founder mice were screened by using the polymerase chain reaction with transgene-specific primers (for a detailed description of transgenic procedures, see Ref. [8]).

2.1.2. Group 2: Nonfunctional CRF transgenic animals

This group consisted of two subgroups of 13 (Group 2a: 8 females and 5 males; 6 litters) and 20 (Group 2b: 7 females and 13 males; 6 litters) transgenic mice with a nonfunctional CRF construct. In the nonfunctional construct, no promoter was present, and therefore no expression of the construct could occur. This group of animals was included in order to investigate the sole effect of integration of foreign DNA into the genome. The fragment was microinjected into the male pronucleus of fertilized eggs (C57BL/6N Crl) and the same procedure was performed as described for Group 1; each subgroup was derived from a different founder male (nonfunctional CRF construct), which was crossed with a wild-type C57BL/6N Crl female.

2.1.3. Group 3: Nontransgenic animals after transgenic procedures

This group consisted of two subgroups of 26 (Group 3a: 11 females and 15 males; 7 litters) and 7 (Group 3b: 3 females and 4 males; 1 litter) mice. The animals in Group 3a received the same treatment as Group 1, but the transgene could not be detected in the progeny. The animals in Group 3b were derived from zygotes, manipulated in the same way, but without a gene construct in the injection needle (the male pronucleus was penetrated, but nothing was injected).

2.1.4. Group 4: Control animals

These animals were normal C57BL/6N Crl animals, not subjected to any transgenic procedure. A total of 26 mice (17 females and 9 males; 4 litters) were tested.

No significant difference in litter size between the treatment groups and controls were found (mean 6.6 pups per litter), as previously described [7]. Each litter is from a different mother. For Groups 1a, 1b, 2a, and 2b, only the transgenic animals were tested.

2.2. Housing

After weaning, at the age of 3–4 weeks, animals of the four groups were maintained as siblings, separated according to sex. They were housed in groups of two to three animals in wire-topped elongated Macrolon Type II cages (530 cm^2; 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 with weekly cage cleaning. Animals were housed conventionally and maintained under standard conditions (12-h light/12-h dark cycle with lights on from 0600

to 1800 h, room temperature 19–25°C, relative humidity 40–70%). Food pellets (RMH-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 analyzed for all groups for the whole test period.

2.4. Behavioral tests

During the 6-month study, animals were subjected individually to several behavioral tests from weaning onwards. The tests were used to compare different aspects of behavior.

2.5. Hole board test (exploration and habituation)

Exploratory behavior was studied in a 16-hole board task [9]. The apparatus used has been described by Van de Weerd et al. [10]. The test was performed twice, to study habituation as well. The number of holes explored during 3 min of testing was counted. A dip was registered 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, the frequency of rearing at the walls of the lid, grooming, and feces and urine production was also registered for each mouse.

2.6. Cage emergence test (escape from novel environment)

In the cage emergence test [10], a mouse is placed into an unfamiliar cage (Macrolon Type I cage, size 204 cm^2 with a hole, measuring 4 cm in diameter, in one sidewall, no lid on top), with its back to the opening. Its reactivity to escape (latency in seconds) from this novel environment (with all four feet outside the cage) onto the table is measured. During testing, frequency of rearing at the walls of the cage, sniffing at the hole, freezing, grooming, and feces and urine production were also recorded.

2.7. Behavioral profile as registered by LABORAS™

A newly developed behavior registration system LABORAS (Laboratory Animal Behavior Observation, Registration and Analysis System, Metris, Hoofddorp, The Netherlands) for the automated registration of different behavioral elements [11,12] was used for the following studies: (a) 24-h behavior observation, (b) 12-h extra climbing behavior, and (c) 10-min light–dark test. With a specially designed sensing platform, the position and the six behavioral categories — immobility (“sleeping”), locomotion, grooming, climbing, eating, and drinking — can be deduced from the vibration patterns evoked by individually housed mice in a cage during a prolonged period of time without disturbing the animal. LABORAS registrations were validated by comparing them with data from observations of videotapes by human observers [13].

Four animals of a group could be tested simultaneously using four different platforms. Each mouse was placed individually in a (clean) Macrolon Type III cage (840 cm^2, with bedding) on the sensing platform. The mechanical vibrations caused by the animals’ movements are transformed into electrical signals and recorded. The signals are “translated” into the six separate behavioral categories and automatically registered by a computer. Signals not recognized by LABORAS are classified as “undefined” ($< 10\%$). Introduction of a mouse in the LABORAS system always took place between 1600 and 1700 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.

2.7.1. The 24-h behavior observation

During 24 h, the behavior of a mouse was recorded to study the treatment effect on circadian rhythms and time budgets of the animals. For analyses, these 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. Per observation period, the relative mean time (mean percentage of time spent on each behavioral category) was calculated and analyzed.

2.7.2. The 12-h extra climbing behavior

Directly after the 24-h test, a metal climbing grid (size 16×10 cm, mesh size 0.5×0.5 cm) was vertically attached to the cage lid, to study differences in climbing behavior after enrichment for the following 12 h (during the dark period). The relative mean time spent on climbing behavior with this extra climbing object in the four time periods 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.

2.7.3. Light–dark test (index of anxiety)

Anxiety-related behavior was investigated in a light–dark test [14] using a cage specially adapted for LABORAS (Macrolon Type III cage, $38 \times 22 \times 27$ cm, two equally sized compartments, one illuminated by 1000 lx). A clear Perspex tunnel ($10 \times 6 \times 5$ cm) connected the dark with the light compartment. At the start of the experiment, each group was subdivided into two equal subgroups. Mice of one subgroup were placed in the dark compartment of the cage, and of the other subgroup in the light compartment. For the next 10 min LABORAS recorded the position of the animals.

2.8. Response to handling (at and after handling)

This part 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 1600 and 1700 h. The procedure of testing was standardized as follows: a mouse was taken out of the cage, put on the table, and held by its tail with one hand, while with the other hand, a mark was placed on the tail. A score was given for the behavioral response during this procedure. The scores used ranged from one to seven, as described by Van de Weerd et al. [15]. Several other responses of the animals (biting, freezing, or urine/feces production) were also scored during the manipulation. Subsequently, the behavior of the animals was observed in their home cage for 10 min using an instantaneous sampling method, meaning that every 5 s behavior of the animal was noted according to a predefined ethogram, based upon Blom et al. [16]. The following behaviors are distinguished: immobility, locomotion, rearing, grooming, digging, climbing, eating, social behavior, and fighting.

2.9. Postmortem examinations

At the end of this study, the animals were killed and postmortem macroscopic inspection was carried out using six males and six females (chosen at random) of each group (except for Group 3b where all seven animals, four males and three females, and Group 2a where five males and seven females were examined). Subsequently the heart, kidney, spleen, and liver were removed, blotted dry, and weighed.

3. Statistics

All data were statistically analyzed using SPSS for Windows 9.0, to determine significant differences between the treatment groups and the control group. Where appropriate, variables were transformed using the natural logarithm or square root transformation to promote homogeneity of variances and normality of the data. The body weight results (mean body weight and growth rate) were analyzed by repeated-measurements analysis of variance (between and within 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 light–dark test and the organ weights. The results of the cage emergence test were analyzed by one-way ANOVA. If ANOVA showed significant effects with respect to the behavioral measurements, treatment groups were compared to the control Group 4 by using Dunnett's 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 analyzed using nonparametric statistics, i.e., the Kruskal–Wallis test, followed, if significant, by the Mann–Whitney U test with Bonferroni correction. The level of statistical significance was preset at $P < .05$ for all parameters. All data are presented as mean values ± S.E.M. If sex differences were not statistically significant, data from male and female mice were pooled.

4. Results

4.1. Survival rate

No major effects of the different transgenic treatments were found on survival rates. In both Groups 1 and 2, three mice died during the test period, while none died in Group 3 and the controls. However, the death of two males of Group 1 was probably due to fighting with their cage mates (wounds were found both on their back and genitals). The cause of death of the females is not known. No major pathology was found during postmortem examination of these mice.

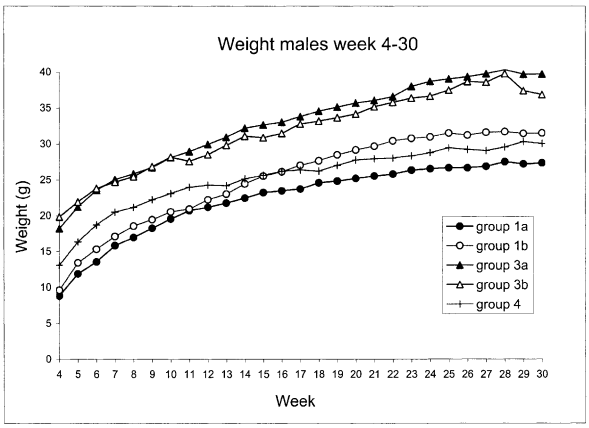
4.2. Body weight/growth rate

Significant differences were found between groups [$F(6,120) = 43.26$, $P < .001$] and gender [$F(1,120) = 142.34$, $P < .001$] for body weight. Overall, males weighed significantly more than females ($P < .001$) for all groups. There was no overall significant difference in mean body weight of the CRF transgenic mice of Groups 1a and 1b compared to the control Group 4, with the exception of the males of Group 1a ($P < .001$). However, the weight gain of the mice of Groups 1a and 1b was significantly faster than the control Group 4 ($P < .001$), especially for the females of Group 1b. This was due to the fact that after weaning (Week 4) and during the following 10 weeks of the test period, the mean body weight of both males and females of Groups 1a and 1b was lower than the controls, while from the age of 15–18 weeks, these mice became heavier than the controls, except for the males of Group 1a (see Fig. 1).

No significant differences in mean body weight and growth rate were found for both males and females of Groups 2a and 2b compared to the control group during the test period (omitted in Fig. 1 for clarity). Also, no significant differences in growth rate were found for the mice of Groups 3a and 3b compared to the control group; however mean body weight of both males and females was significantly higher compared to the controls for the whole test period ($P < .01$).

4.3. Morphology/clinical appearance

All the CRF transgenic mice (Groups 1a and 1b) showed differences in morphology and clinical appear-



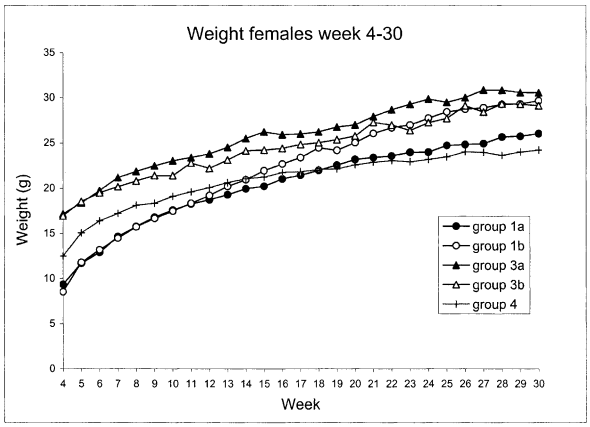


Fig. 1. Mean male (a) and female (b) bodyweight (g) in postweaning period (age 4–30 weeks). No significant differences were found in mean bodyweight or growth rate between mice of Groups 2a and 2b compared to the control group (data not shown, omitted for clarity).

ance: the shape of the head was broader and shorter than in control animals. They also showed features of Cushing's syndrome, such as hair loss and thinner hair on both their head and back. These differences were already present in the preweaning period [7]. No differences in morphology or clinical appearance were detected for the other groups.

4.4. Exploratory behavior

A significant effect of group [$F(6,123)=43.84$, $P<.001$] on the number of holes explored in 3 min was detected. Fig. 2 shows that in both hole board tests the transgenic CRF animals (Groups 1a and 1b) explored significantly fewer holes compared to the control group ($P<.001$) or any other group ($P<.01$). For all groups, effects for males and females were similar, although overall females explored more holes than males [$F(1,123)=5.23$, $P<.05$]. There was a significant difference in number of holes explored in both tests between Groups 3a and 3b (Test 1: $P<.01$; Test 2: $P<.001$). All groups, except Group 2b, showed a significant decrease in number of holes explored in Test 2 compared to Test 1 [$F(1,123)=52.57$, $P<.001$]. During both tests, the transgenic CRF animals showed significantly more rearing to the walls of the transparent lid compared to the other groups, especially Group 1b ($P<.002$). During the first hole board test, the feces production of the Groups 1a, 1b, and 2b was significantly higher than the control group ($P<.01$).

4.5. Cage emergence test

For the cage emergence test (Fig. 3), no significant differences in time to escape from the empty cage were found between the different treatment groups and the control group. No differences were found between males and females for all groups, nor for the various behaviors scored during the test. The behaviors most frequently observed were rearing to the sidewalls and sniffing at the hole.

4.6. Light–dark test

In the light–dark test, the latency to leave the compartment for the first time, as well as the times spent in the light or in the dark compartment and the mean number of movements from the light to the dark compartment, and vice versa (crossings), were recorded. The results revealed differences between the transgenic CRF animals and the control group for each of these four parameters, regardless

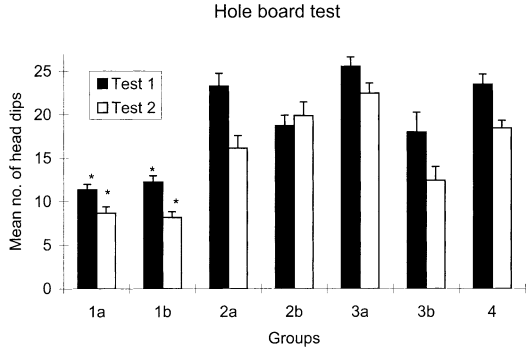


Fig. 2. Exploratory behavior of the four test groups in two subsequent hole board tests. Group 1: CRF transgenics with functional CRF construct (1a and 1b represent progeny from two different founders), Group 2: CRF transgenics with nonfunctional CRF construct (2a and 2b represent progeny from two different founders), Group 3: nontransgenics after transgenic procedures (3a: injected with DNA construct, no integration; 3b: transgenic procedure, but no construct injected), and Group 4: control animals (no transgenic treatment). Testing was performed at the age of 12 weeks (Test 1) and 14 weeks (Test 2) between 1500 and 1700 h. Test period was 3 min. Data are expressed as mean numbers of head dips ± S.E.M. Significance ($P<.05$) based on ANOVA (repeated measurements) with main between-subject factors groups and sex and main within-subject factors Tests 1 and 2. Dunnett's post hoc tests with Bonferroni correction were used to study differences within groups. *$P<.001$, significant difference compared to control group.

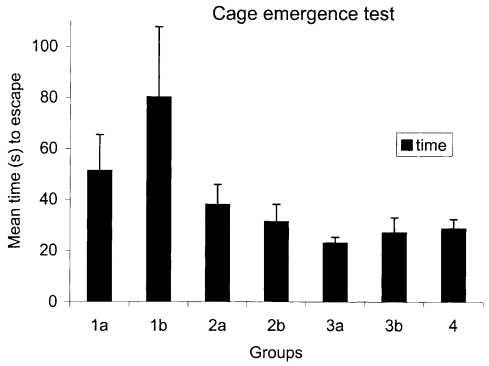


Fig. 3. Mean time (±S.E.M., in seconds) to escape from an empty cage. Testing was performed at the age of 16 weeks between 1500 and 1700 h. Maximum testing period was 10 min. Differences between treatment groups and control group analyzed by one-way ANOVA (ns).

whether the test started by placing the animals in the dark or in the light compartment [$F_{latency}(6,123) = 15.34$, $P < .001$; $F_{light}(6,117) = 2.18$, $P < .01$; $F_{dark}(6,117) = 3.69$, $P < .01$; $F_{crossings}(6,122) = 19.99$, $P < .001$; respectively, see Fig. 4). Gender effects were only observed for the number of crossings [$F(1,122) = 10.13$, $P < .01$].

Latency until the first entry in the other compartment was increased for Groups 1a and 1b when compared to controls ($P < .001$) and to the other groups in both tests (1a vs. 2a, 2b, 3a, 3b, and 4: $P < .01$; 1b vs. 3a, 3b, and 4: $P < .001$). Overall, when started from the light compartment, latency is higher (Fig. 4a) than when started from the dark compartment (Fig. 4b). This difference is mainly caused by the transgenic mice of Groups 1a and 1b ($P < .01$, overall 44% higher).

Mice from all groups showed a preference for the dark compartment, as measured by total time spent in the dark vs. in the light during the 10-min test sessions, when starting from the dark compartment (Fig. 4b). Total time spent in the light is only significant shorter for Groups 1a and 1b compared to the control group ($P < .01$) and the other groups ($P < .05$).

When starting from the light compartment (Fig. 4a), the time spent in the light and dark compartment tends to be equal with an exception for Group 1 (Groups 1a and 1b spending more time in light compartment than the control group, $P < .01$).

Overall, the number of crossings (Fig. 4c) was significantly less for the transgenic CRF animals compared to the control group, especially when the animals were first placed in the light compartment ($P < .001$). For the other groups, no significant differences could be demonstrated in number of crossings compared to the control group. For all groups, effects for males and females were similar, although overall females showed more crossings than males ($P < .01$, overall mean 20.9 vs. 16.5). Starting from the dark compartment resulted in more crossings for all groups compared to starting from the light compartment (overall mean of 3.3 more crossings, $P < .05$).

4.7. LABORAS 24-h test

Fig. 5 presents the results of the 24-h behavior as recorded by LABORAS. Per time period, the mean percentage of 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 time, is not shown. Lights went out in the 1–3-h period and on again in the 13–15-h period. Gender effects were only present for climbing behavior. Significant differences in behavioral patterns were found between transgenic CRF animals (Group 1b) and the control group (mainly during the dark period).

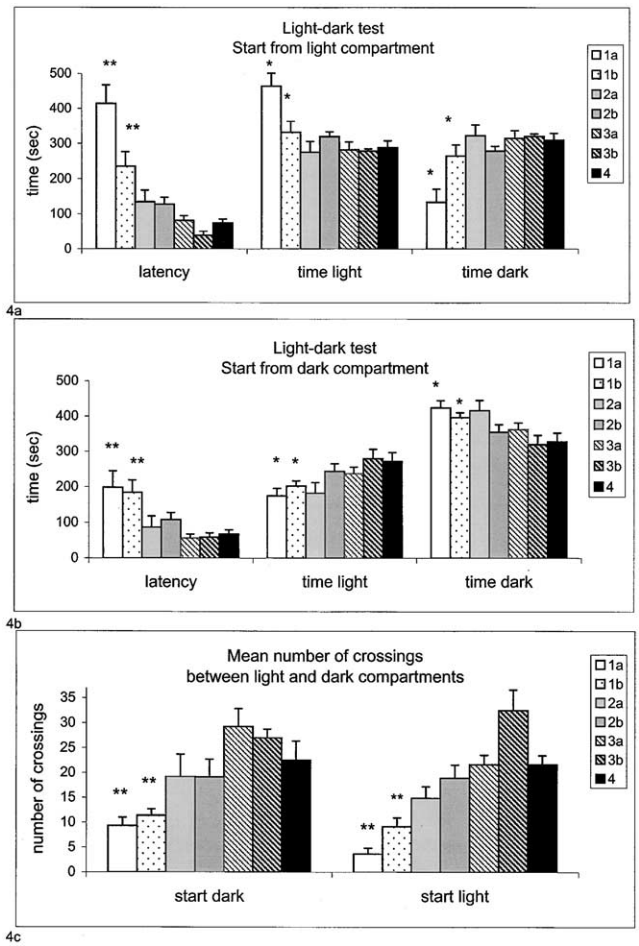


Fig. 4. Results of the light–dark preference test. (a) Light–dark test, start from light compartment, (b) light–dark test, start from dark compartment, (c) mean number of crossings between the two compartments. Half of the number of the animals of each group was placed in the light compartment (a) and the other half in the dark compartment (b) at the start of the experiment. Latency (in seconds) to first entry (entry scored when mice is with all four paws in the other compartment), total time spent in light and dark compartments (in seconds), and number of crossings (c) of the light–dark test (mean ± S.E.M.) are shown. Testing was performed at the age of 20 weeks between 1500 and 1700 h. Test period was 10 min. Significance ($P < .05$) based on two-way ANOVA with main factors group, sex, and input in dark or in light compartment. $*P < .01$; $**P < .001$, significant difference compared to the control group.

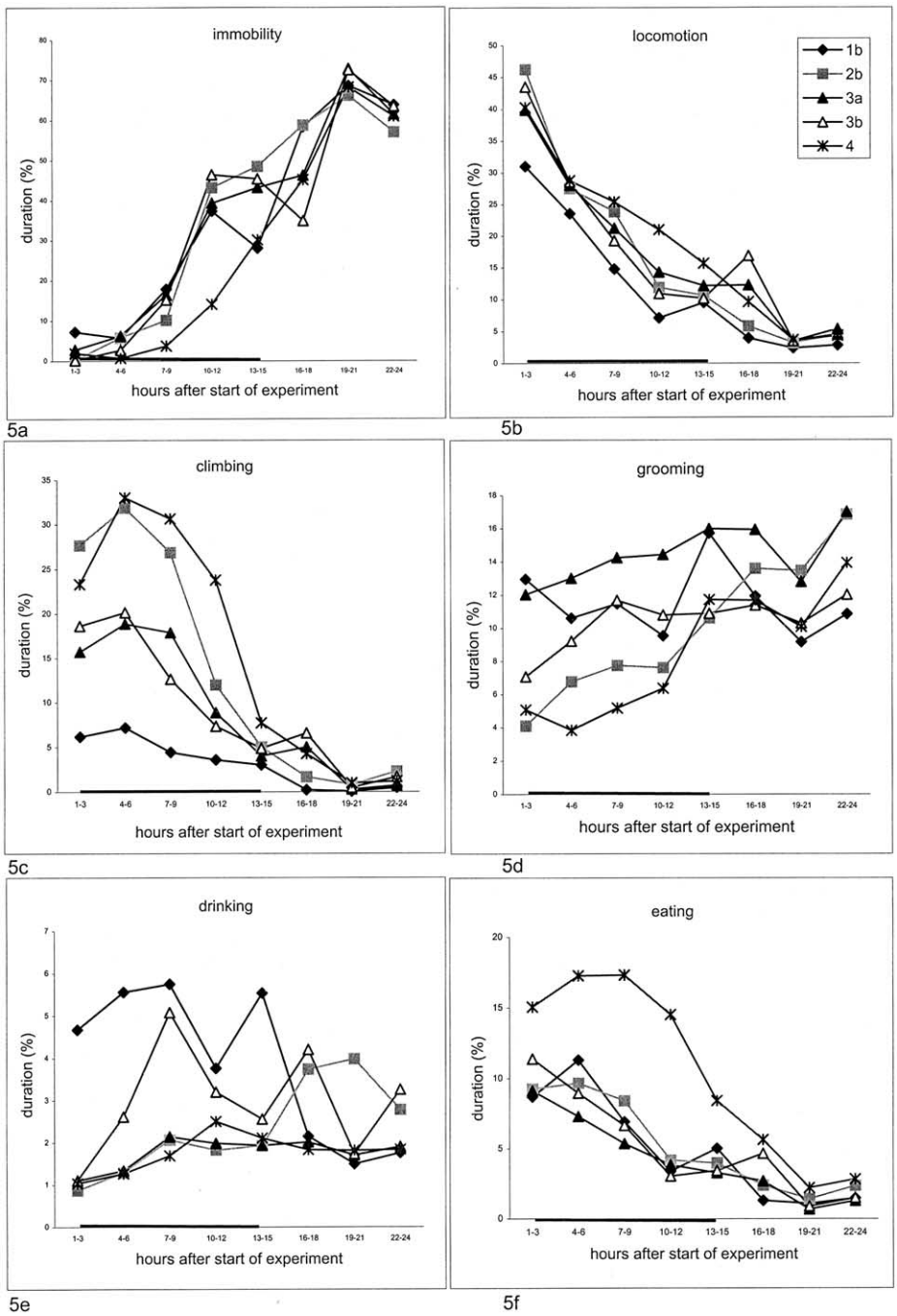


Fig. 5. Results of the LABORAS behavior registration system, 24-h test. Per time period of 3 h, the relative mean time spent on each of the six behavioral categories are shown for mice of Groups 1b, 2b, 3a, 3b, and 4 during 24 h of testing. The data of the category ''undefined'' are not shown. Lights 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. Significance ($P<.05$) based on ANOVA (repeated measurements) with main between-subject factors group and sex and main within-subject factors the eight time periods. Dunnett's post hoc tests with Bonferroni correction were used to study differences within groups.

4.8. Immobility (Fig. 5a)

Overall, all groups [$F(4,86)=6.74$, $P<.001$] except Group 3b, spent more time on immobility than the control animals (1–24 h, Groups 1b and Group 3a: $P<.005$, Group 2b: $P<.05$). For the first two periods (1–3 and 4–6 h), only the CRF transgenic animals showed higher percentages of immobility ($P<.05$ and $P<.01$), while for the 7–9- and 10–12-h periods, the immobility of the other groups was also significantly higher compared to the controls ($P<.005$). During the light period, no significant differences in duration of immobility were found between the groups.

4.9. Locomotion (Fig. 5b)

The transgenic CRF animals showed significant less locomotion compared to the control group, mainly during the dark period [ANOVA group effect: $F(4,86) = 10.25$, $P < .001$; Group 1b vs. controls: $P < .01$].

4.10. Climbing (Fig. 5c)

Overall, the CRF transgenic mice spent significant less time climbing compared to the control group [ANOVA group effect: $F(4,86) = 25.04$, $P < .001$; 1–24 h, Group 1b vs. controls: $P < .005$]. In the first four periods (dark period), the mice of Group 3a also showed less climbing behavior ($P < .05$) compared to the control group; the difference between the two groups is smaller than in the case of the transgenic mice. No significant differences between Groups 2b and 3b and the control animals were found. Overall, females spent more time on climbing than males for all groups [$F(1,86) = 49.70$, $P < .001$].

4.11. Grooming (Fig. 5d)

Overall, ANOVA revealed a significant group effect for grooming [$F(4,86) = 4.08$, $P < .01$]. For the 1–3-, 4–6-, and 7–9-h periods, animals of Group 1b ($P < .05$) and Group 3a ($P < .01$) showed significant more grooming compared to the controls, while for the 10–12-h period, this was only significant for Group 3a ($P < .01$). For the other periods, no significant difference was found.

4.12. Drinking (Fig. 5e)

For the 1–3- ($P < .005$), 4–6- ($P < .005$), and 7–9-h ($P < .01$) periods, the CRF transgenic mice showed significant more drinking behavior compared to the control group [ANOVA group effect: $F(4,86) = 3.01$, $P < .05$].

4.13. Eating (Fig. 5f)

All groups of mice showed a significantly reduced eating behavior when compared to the control group [ANOVA group effect: $F(4,86) = 15.40$, $P < .001$; Group 1–3 vs. controls: $P < .005$]. This was most evident during the dark period ($P < .05$).

4.14. LABORAS 12-h extra climbing

Directly after the 24-h test, the 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 [$F(1,86) = 25.54$, $P < .001$]. This difference is less significant for the transgenic mice of Group 1b, where both males and females showed very little climbing behavior (data not shown). Climbing behavior decreases during the four periods for all males when climbing of the 12-h test is compared to the 24-h test. However, the females, especially the females of Group 2b showed increased climbing behavior for the 4–6- and 10–12-h periods. Overall, mice of all

Table 1
Absolute organ weights expressed as mean (±S.E.M.) grams $\times 10^{-2}$, split by group and gender

Organ	Group 1a	Group 1b	Group 2a	Group 2b	Group 3a	Group 3b	Group 4
Bodyweight							
Males (g)	24.99 ± 1.62*	30.41 ± 1.76	38.02 ± 2.84	35.79 ± 1.84	43.70 ± 1.76***	42.45 ± 1.18***	31.77 ± 0.93
Females (g)	23.31 ± 0.54	33.02 ± 1.76*	34.08 ± 3.65*	27.54 ± 1.76	33.65 ± 1.25*	33.06 ± 0.79*	24.63 ± 0.57
Spleen							
Males	5.00 ± 0.45**	5.50 ± 0.22**	7.60 ± 0.60	8.50 ± 0.50	7.33 ± 0.56	7.50 ± 0.65	10.83 ± 2.5
Females	5.00 ± 0.52**	6.17 ± 0.40**	8.71 ± 0.68	13.67 ± 2.20	10.17 ± 0.95	9.00 ± 0.69	19.17 ± 0.75
Heart							
Males	14.00 ± 0.93	16.33 ± 0.99	18.20 ± 0.66	17.33 ± 0.84	16.33 ± 0.49	16.00 ± 0.71	16.00 ± 0.77
Females	13.67 ± 0.56	13.33 ± 0.49	14.57 ± 0.81	14.67 ± 0.92	15.50 ± 0.62	14.00 ± 0.58	12.50 ± 0.43
Kidney							
Males	18.00 ± 1.82	20.17 ± 1.01	26.60 ± 1.72*	25.17 ± 1.30*	25.17 ± 0.60*	24.75 ± 0.63*	22.33 ± 1.09
Females	17.67 ± 0.56	18.83 ± 0.79	19.86 ± 1.39*	21.33 ± 0.99*	22.67 ± 1.20*	21.33 ± 1.20*	16.67 ± 0.80
Liver							
Males	115 ± 11.13	149 ± 11.31	194 ± 16.8*	185 ± 13.50	226 ± 34.35*	218 ± 15.72*	158 ± 12.97
Females	125 ± 4.29	151 ± 6.44	165 ± 13.22*	126 ± 10.82	147 ± 10.96*	159 ± 5.78*	120 ± 5.74

Values are means of six males or six females per group (±S.E.M., in grams $\times 10^{-2}$), except for Group 2a (five males and seven females) and Group 3b (four males and three females). Bodyweights are shown in grams. Significance ($P < .05$) based on two-way ANOVA with main factors group, sex, and absolute or relative organ weight.

* $P < .05$, significant difference compared to the control group.

** $P < .01$, significant difference compared to the control group.

*** $P < .001$, significant difference compared to the control group.

groups did not spend significantly more time on climbing when the extra climbing grid was added to their cage.

4.15. Handling test

The score of the response to handling and the behavior after handling scored for 10 min in the animals' home cage did not reveal any significant difference between the groups.

4.16. Postmortem examinations

Significant differences were found for the adult body weight between groups [$F(6,65) = 14.59$, $P < .001$] and gender [$F(1,65) = 25.99$, $P < .001$] at the postmortem examination (Table 1, see also Fig. 1). The males of Groups 3a and 3b were heavier compared to the controls ($P < .001$) and the males of Group 1a were lighter ($P < .05$), while the females of Groups 1b, 2a, 3a, and 3b 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 (grams per total body weight).

Overall, we found a statistically significant decrease in absolute weight of the spleen of Groups 1a and 1b for both males and females compared to the controls [ANOVA group effect: $F(6,65) = 8.02$, $P < .001$; Groups 1a and 1b vs. controls: $P < .01$]. After analysis of the relative spleen weight, this decrease was also significant for males of all other groups [ANOVA group effect $F(6,32) = 3.41$, $P < .05$; Group 1–3 vs. controls: $P < .05$], except Group 2b. No absolute heart weight differences were found, but relative heart weight of the males of Group 3 was significantly lower than controls [ANOVA group effect: $F(6,32) = 5.46$, $P < .01$; Group 3 vs. controls: $P < .05$]. For the relative weight of the kidney and the liver, no significant differences were found between groups and gender. Absolute kidney weights were increased for Groups 2 and 3 [ANOVA group effect: $F(6,65) = 8.94$, $P < .001$, Groups 2 and 3 vs. controls: $P < .05$] and absolute liver weights were increased for Groups 2a and 3 compared to the controls [ANOVA group effect: $F(6,65) = 5.93$, $P < .001$: Groups 2a and 3 vs. controls: $P < .05$].

Postmortem macroscopic autopsy revealed no obvious differences between the various groups. Overall, males had a smaller adrenal gland compared to the females for all treatment groups. Some tumor development was detected in a few females (Group 1a: one subcutaneous, Group 2a: one in the pancreas, Group 2b: one in the lungs) and one male (Group 2a: in small intestine). In two females of the control group, enlarged lymph nodes were found in the flank/belly of the animal.

5. Discussion

This study evaluated the impact of biotechnological procedures involved in the production of transgenic animals on the welfare of these animals during their postweaning development by measuring various behavioral, physiological, and anatomical/morphological parameters.

At weaning, the average body weight of mice was lower in Groups 1a and 1b, while it was higher in Groups 3a and 3b compared to the control group. To recover from underweight, the pups of the CRF transgenics received extra mashed food, which was daily added to their cages for a period of 2 weeks directly after weaning. During the first weeks of the postweaning period, the mice of Group 1 showed lower body weights, but from age 15–18 weeks on, they became heavier than the controls (except the males of Group 1a).

The animals of Group 3 were heavier during the whole postweaning period than the controls, but they did not show a higher growth rate. The transgenic animals with a nonfunctional construct (Groups 2a and 2b) did not differ significantly in their body weight from the controls during both the pre- and postweaning period.

All the transgenic CRF mice showed features of Cushing's syndrome, such as hair loss and a thin skin, alopecia (baldness), and truncal obesity (in some mice) and a different shaped head (broader and shorter than wild-type mice). Several of these features are obviously due to increased corticosterone levels, caused by the overexpression of the CRF gene [17,18].

The hole board test represents a novel environment of increased structural complexity [19]. It is designed to test exploratory behavior, as it takes advantage of the natural tendency of mice to dip their heads into holes [14, 20]. In both hole board tests, the transgenic CRF mice were hypoactive compared to all other groups. During the first test, they also produced more feces than control mice, altogether indicating a higher state of anxiety.

All groups (except Group 2b) showed a decrease in dips in the second hole board test. This is in line with the results of Dorr et al. [21], who also found in a comparable test a reduction in number of head dips in the second test. The mice were less active and more hesitant, sniffed more, and walked less deliberately. The authors regarded this as a sign of reduced curiosity or habituation. It might indicate that explorative behavior is diminishing with time. Apparently, in the present study, there are no differences in habituation between the different treatment groups.

No significant differences in reactivity were found in the cage emergence test for all groups, but the transgenic CRF mice showed a greater variation in time to escape from the novel environment (not significant). Although all animals escaped from the cage within 10 min, more animals in Group 1 escaped after 60 s than in the other groups, also indicating enhanced anxiety.

The light–dark test has frequently been used to test anxiolytic action of new drugs [22,23]. In the present study, the CRF transgenic mice seemed to be more anxious than animals of other groups (longer latency to enter the other compartment, less number of crossings, and less time spent in the light compartment).

The choice to move from dark to light confronts the animal with a conflict situation between the drive to explore the new environment and the aversion for bright light [24]. An unexpected finding was that, if animals were placed in the light compartment, more crossings and shorter latency were seen than if animals were placed in the dark compartment. Overall, mice placed in the light compartment spent equal time in the light and dark compartment, while mice placed in the dark compartment spent less time in the white compartment, having a preference for the dark enclosed space.

The results of the 24-h behavior observations showed similar behavioral circadian patterns for Groups 2–4. High levels of activity associated with exploration [25] were observed in the first 3 h (cf. locomotion, 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 [12,13,26]. The CRF transgenic animals (Groups 1a and 1b) showed a similar pattern but spent less time on locomotion and climbing and more time on immobility than the other groups. During the dark period, drinking was increased (polydipsia), which is a common feature of animals with Cushing's syndrome [27,28].

The control mice showed more eating behavior compared to the other groups, although their bodyweight did not increase. Eating behavior as scored by LABORAS includes gripping the bars of the food hopper and gnawing the food between the bars, and, therefore, is not necessarily related to food consumption.

No significant differences in responsiveness to handling were found for all groups. This could be due to the fact that all animals were frequently handled from birth and were therefore more used to handling routines. Also, the transgenic CRF mice showed no increased (stress) response to handling.

No major pathology was found during postmortem examinations. The absolute weight of the spleen was decreased for the transgenic CRF mice compared to the control group. Boehme et al. [18] also found that after adrenalectomy, the size and weight of the spleen of transgenic CRF mice recovered to nearly normal.

6. Conclusion

The tests described in this paper were primarily designed to estimate the effects of biotechnological procedures of transgenesis on the development of the animals in the postweaning period (Weeks 4–30) of mice. In the present study, differences in behavior, bodyweight, and morphology between animals with CRF transgenic expression (Group 1) and the control group were found. Behavior of transgenic CRF mice differed significantly from control animals in test situations designed to assess behavioral activation and anxiogenic-like states. They showed less exploratory behavior in the hole board test, more hesitation to escape from a novel environment and more anxiety in the light–dark test. During the 24-h individual observation test, they showed less locomotion, more immobility, and less climbing behavior. No significant differences for the other treatment groups (Groups 2 and 3) were found. The biotechnological procedures of microinjection seem to have no major effect on the normal development and thus on the welfare of the mice. Previous results, obtained by screening animals during Days 0–21 (preweaning period) substantiate these observations [7]. About 10% of the animals with a DNA construct did not survive the first 2–3 days after birth. It might be that this is the reason why relatively few welfare problems have been observed in Group 1 (except those related to CRF expression) and Group 2. However, in this respect, general conclusions can only be drawn when more and different transgenic lines have been studied this way.

Most of the behavioral tests as employed in this study seem to be sufficiently discriminative to differentiate between the treatment groups and controls. Thus, these tests can also be used for behavioral phenotyping of other newly produced transgenic lines and to study possible effects on their welfare.

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