TRANSGENESIS AND ANIMAL WELFARE

Implications of transgenic procedures for the well-being of the laboratory mouse

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Gevolgen van transgenese technieken voor het welzijn van muizen

(met een samenvatting in het Nederlands)

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Voor Jeroen en baby Roos
Voor mijn ouders

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Chapter 1

Introduction

Parts of this introduction have been published elsewhere (van der Meer et al. 1996, 1997).

Rationale for studying welfare aspects of transgenic animals

Transgenesis can be defined as the stable incorporation, by using artificial gene transfer, of exogenous DNA into the genome of an organism, in order to introduce or delete specific characteristics of the phenotype (Mepham et al., 1998). The first transgenic experiments in mammals were performed in mice (Gordon et al., 1980; Gordon and Ruddle, 1981). The production of a mouse provided with a gene for rat growth hormone in its genetic material, resulting in an animal twice the normal size (Palmiter et al., 1982), was the start of a widely used method to produce transgenic animals. Most of the transgenic experiments are still carried out with mice. The mouse is a very suitable model for these studies because: 1) much information is available on mouse genomics; 2) adequate techniques for culturing and handling embryos and embryonic stem cell lines have been developed; 3) inbred strains with specific genetic and phenotypic characteristics are available; 4) mice are easy to handle, and 5) mice have a short generation interval. In addition, the mouse shares most metabolic pathways and physiological processes with the human species (Verbeek, 1997). Results obtained with transgenic mouse models come from various research areas: the molecular biology of gene regulation, immunology, developmental biology, oncology and human disease models (Lathe and Mullins, 1993; Buehr and Hjorth, 1994; Monastersky and Robl, 1995; Bedell et al., 1997). The novel methods of genetic modification also provided opportunities to introduce specific changes into the genome of farm animals. Although the efficiency is still low, successful gene transfer has been reported for pig, sheep, rabbit, goat and dairy cattle (Ebert and Schindler, 1993; Pursel and Rexroad, 1993; Bawden et al., 1995; Schmoekel et al., 1997).

Through transgenesis more refined animal models can be obtained than with genetic changes achieved by traditional breeding methods. Genetic engineering is not a simple extension of previously existing techniques. It allows not only the insertion into the genome of new or extra copies of genes, which may be derived from a different species, but also the "knockout" of targeted genes (Hubrecht, 1995). These methods provide very precise tools to investigate the function of a single gene *in vivo*. In contrast to tissue culture experiments, transgenesis offers the chance of studying the interaction of genes within the entire organism. By transgenesis it is also possible to produce animal models of human diseases for which no natural models are available. The increased efficiency of research with transgenic animals may lead to a reduction in the use of animals in specific experiments. There is also the potential for commercial use of the results of transgenic research, such as the production of therapeutic human proteins in the milk of transgenic livestock or rabbits or such as patenting transgenic animals. The first case occurred in 1988 when the “Harvard oncomouse”, a strain that readily develops malignant tumours and which is being used for cancer research, obtained a patent in the USA.

However important these developments are, there is still substantial public concern about the genetic modification of animals through transgenesis. Questions have been raised dealing with the safety aspects of this new development. In particular, fear has been expressed of ecological disasters due to the escape of transgenic animals from the laboratory into the wild. Because genetic material can be exchanged between different species, this has become a major

ethical issue. How far may people go and who is imposing the limitations? Is everything technically possible also acceptable from an ethical point of view? And what about possible welfare problems of transgenic animals? The production of transgenics, involving a large number of animals, is a different approach from the production of mutant animals by traditional breeding. Welfare problems may arise that go beyond those caused by the effect of the genetic trait, which is introduced or changed. Transgenic procedures allow fundamental changes in the genome of animals that could not, or not readily, emerge in nature (Hubrecht, 1995). The two most frequently used techniques for inserting genetic material into the mammalian germ line are pronuclear microinjection and embryonic stem (ES) cell-mediated gene transfer. Especially the random integration of DNA into the genome by microinjection may increase the chance of disturbing normal physiological processes of the animal in an unpredictable way. Therefore, one could argue for an increased chance of welfare problems in transgenic animals when compared to an animal model, in which traits are introduced by traditional breeding methods. The technique of transgenesis is also emerging in a period of time in which the position of animals in relation to man is being reconsidered, or is at least an issue of increasing debate. It is generally recognised that animal use is allowed only if the discomfort for the animals are outweighed by the potential benefits of the experimental results. Consequently, it is necessary to know the impact of transgenesis on the well-being of animals. However, knowledge is lacking concerning the degree of animal's suffering due to transgenic techniques.

Notwithstanding these concerns, according to projections from the OECD (Organisation for Economic Co-operation and Development), research on transgenic mice is expected to continue its drastic growth (see Figure 1) and welfare of the animals is not a main consideration in these experiments.

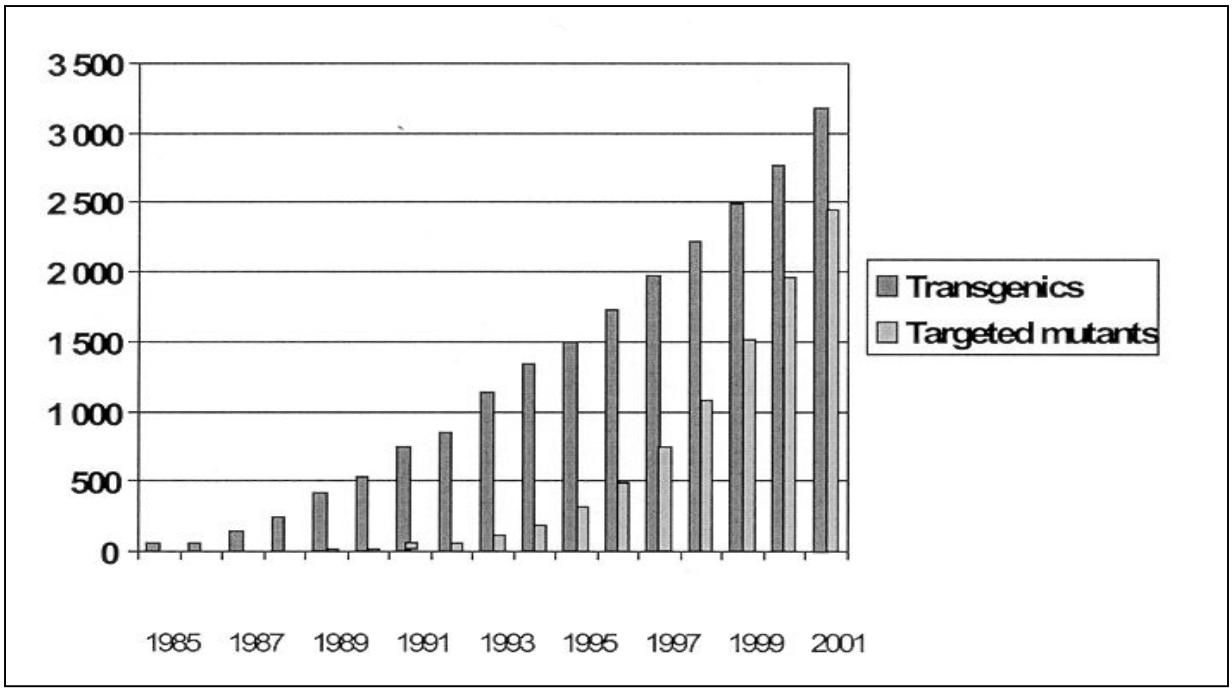

Figure 1: *Number of current and projected publications regarding transgenic and targeted mutant mice (Source: OECD, 1998)*

As the science of genetic engineering continues to evolve, databanks are being created throughout the world to maintain interesting lines of transgenic mice, frozen sperm and embryos, like, for example, EMMA (= European Mutant Mouse Archive), or to provide a source of information on existing transgenic strains, like, for example, TBASE (Transgenic Animal Data Base). Such databanks provide specimens and information to research scientists, which helps to avoid the production of duplicate strains while preserving the existing ones. However, these databanks do not contain information concerning the welfare aspects of the specific strains. This could be extremely useful information for scientists selecting a strain for research, but specific studies concerning the welfare aspects of transgenic animals are still scarce, thus, as yet, little information is available.

Transgenic techniques

To understand the necessity for research into the welfare of transgenic mice and to be able to pinpoint the specific problems that should be addressed, it is vital to know the processes involved in producing transgenic mice. As already mentioned, the two techniques that are most frequently used to produce genetically modified mice are the microinjection technique and the genetic manipulation of embryonic stem cells. Generally, these techniques are used for different purposes. By using microinjection an extra gene can be randomly added to the genome in order to study the expression of new genetic material, while embryonic stem cells are used for site-directed mutagenesis by homologous recombination (Overbeek, 1994). This involves a small modification of an existing gene with the transgene in a precise and predetermined way. In some cases the function of the existing gene is switched off, resulting in "knockout" mice. This has opened new possibilities to re-evaluate a number of fundamental biological questions concerning development and the interaction of cells and organs of higher organisms, in particular to investigate the role of the molecular components of complex *in vivo* systems (Hanahan, 1989).

These two procedures differ quite considerably in their main components and are described separately. The first steps, involving isolation or construction, and purification of the desired DNA construct are similar in both cases. They will not be discussed, as the mode of producing constructs has no direct impact on the welfare of the final product: the transgenic mouse.

Microinjection of fertilised oocytes

The most common and well-characterised approach for producing transgenic animals is to introduce foreign DNA (the transgene) into fertilised one-cell embryos by microinjection. This technique was successfully used for the first time in 1980 (Gordon et al., 1980). Over the last decades, it has become the most widely applied method for gene transfer in mammals. The process of microinjection itself, although technically demanding, is relatively straightforward (shown schematically in Figure 2). The advantage of microinjection is that foreign genes, if equipped with the appropriate regulatory signals, are expressed efficiently. Moreover, there is no apparent limit to the size of the inserted DNA molecule.

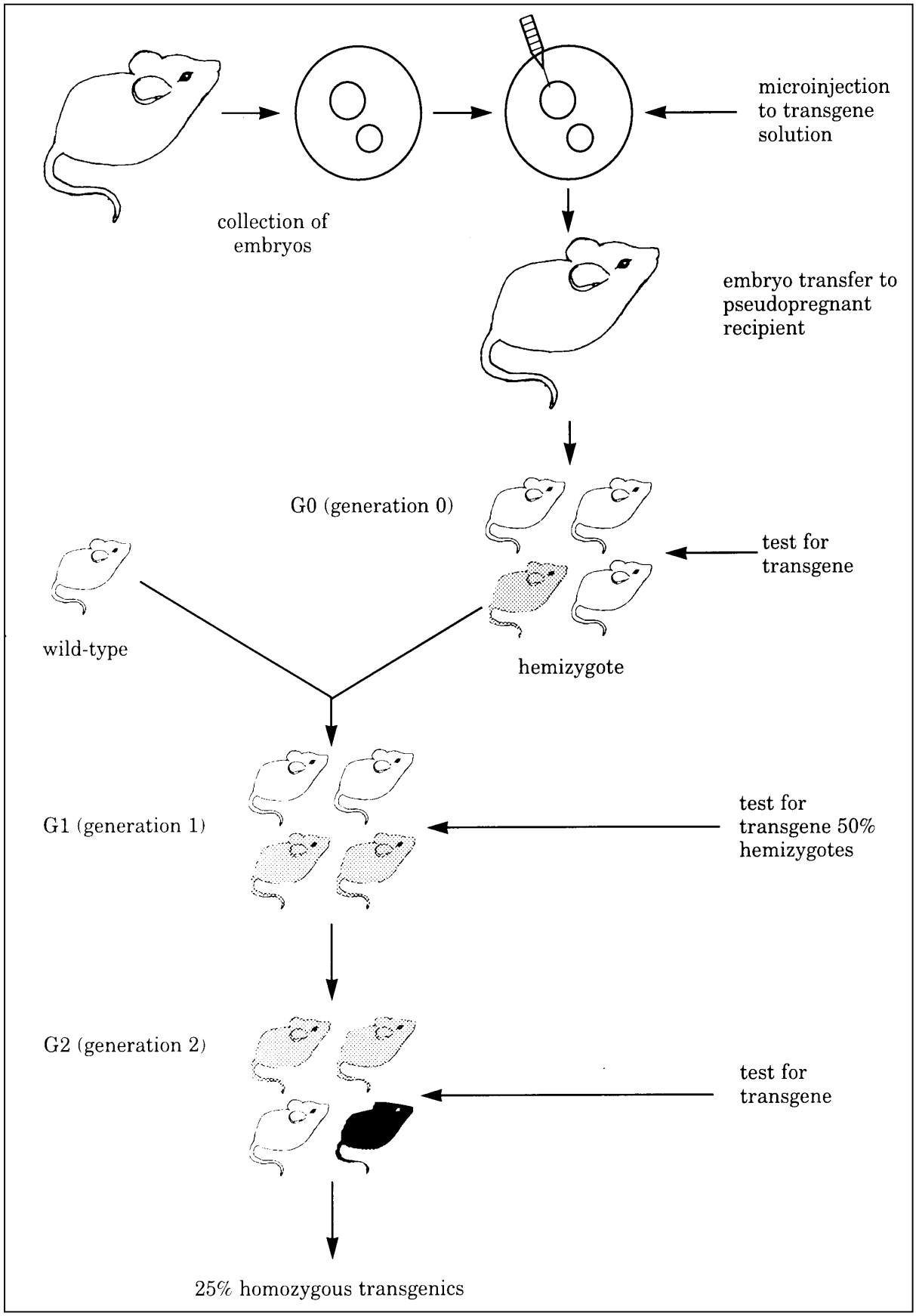

Figure 2: *Microinjection method (after Mepham et al., 1998)*

Egg cell harvesting

To produce fertilised eggs, female mice are hormonally induced to superovulate and mated with syngeneic males. Next morning the females are checked for copulatory plugs and fertilised eggs are collected. A succesfull fertilisation can be checked by observing the two pronuclei under a light microscope. The optimum time for microinjection ranges from the time the male pronuclei at the periphery of the egg membrane are identifiable until the male and female pronuclei merge just prior to the first cleavage (Polites and Pinkert, 1994).

For collection of eggs within this range, the females with a copulatory plug are euthanised approximately 16 hours after mating. The eggs are harvested by dissecting the oviduct. An average of 15 to 30 eggs is harvested per female (Van der Meer, 1994). The eggs are collected in an appropriate medium in a petri dish. Eggs with visible pronuclei and a normal appearance are selected.

Microinjection

A holding pipette is used to immobilise the egg cell and the injection pipette is loaded with DNA. The injection pipette is inserted through the zona pellucida membrane and the pronuclear membrane into the male pronucleus, which is larger than the female pronucleus and therefore easier to manipulate (shown in more detail in Figure 3). The DNA is dispelled into the pronucleus, which is seen to expand. The egg cells remain in culture in a stove until they reach the two-cell stage. The embryos are then ready for implantation in receptive females.

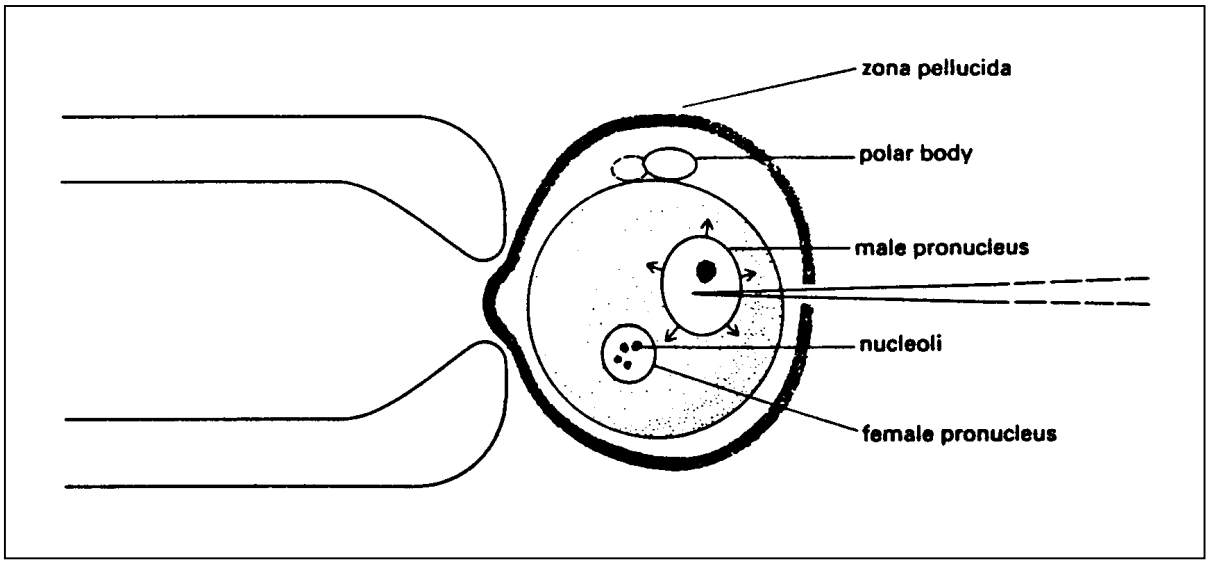

Figure 3: *Microinjection of the pronucleus. The plasma membrane has been pierced, the tip of the needle remains inside the pronucleus. DNA expelled from the needle causes the pronucleus to swell visibly (after Hogan et al., 1986).*

Egg transfer

A second group of female animals are made pseudo-pregnant by mating with vasectomised males. This means that they will be in the correct hormonal state to allow the introduced embryo to implant, but none of their own eggs will form embryos. The injected eggs are then implanted surgically into the oviducts of the recipient mice (approximately 10-20 embryos per mouse) and allowed to develop to term. The recipient females are known as the foster mothers (FM).

Efficiency

The presence of foreign DNA in the offspring is determined by taking a mucosal sample, or a tail or ear biopsy of every pup (at the age of 2-3 weeks) and isolating the genomic DNA. The isolated DNA can be screened for the transgene by several methods (for example Southern blotting or polymerase chain reaction). Although results may vary between different experiments, in most studies the proportion of manipulated embryos developing to term ranges between 10 and 30% and the proportion of this offspring carrying the transgene is usually also between 10 and 30%; thus 1 to 10% of the injected embryos result in transgenic animals (Moore and Mepham, 1995). This initial transgenic offspring are referred to as the founder animals, or in genetic terms, the G_0.

The DNA added to the pronucleus must integrate with the host genome in order to replicate and thus be transmitted to daughter cells. However, the microinjection technique leads to the random integration of the DNA into the genome. If integration occurs prior to the first cell division, all cells in a transgenic pup, including the germ cells will carry the transgene. In practice, a significant proportion (30%) of the transgenic pups is likely to be mosaic, indicating that integration has occurred after the first stage of replication. The mechanism of integration is not known. If the offspring does not carry the transgene in their germ-line cells, this progeny will not produce gametes that pass the transgene on to future generations. By further breeding with the founder mice, stable transgenic lines can be produced.

Gene targeting and gene transfer in embryonic stem (ES) cells

Another approach to the production of transgenic animals (mice) is the use of embryonic stem (ES) cells (shown schematically in Figure 4). ES cells are pluripotent cells, found in the inner cell mass (ICM) of embryos at the blastocyst stage of development. These cells have not yet differentiated and maintain the ability to develop into any type of tissue during the embryonic and foetal development. Transgenes can be introduced into the ES cells (Wilder and Rizzino, 1993; Capecchi, 1989a,b, 1994). The advantage of using ES cells is the opportunity to apply gene targeting thus enabling site-directed insertion of DNA (Moore and Mepham, 1995; Müller, 1997). Gene targeting involves inducing the embryonic stem cell to remove one of its own genes and replace it with a modified version of the same gene. This is achieved by relying on the cell's natural machinery for repairing breaks or faults in the DNA: homologous recombination. Examination of the phenotype of these "knockout" mice can provide a greater understanding of the role this gene normally fulfils.

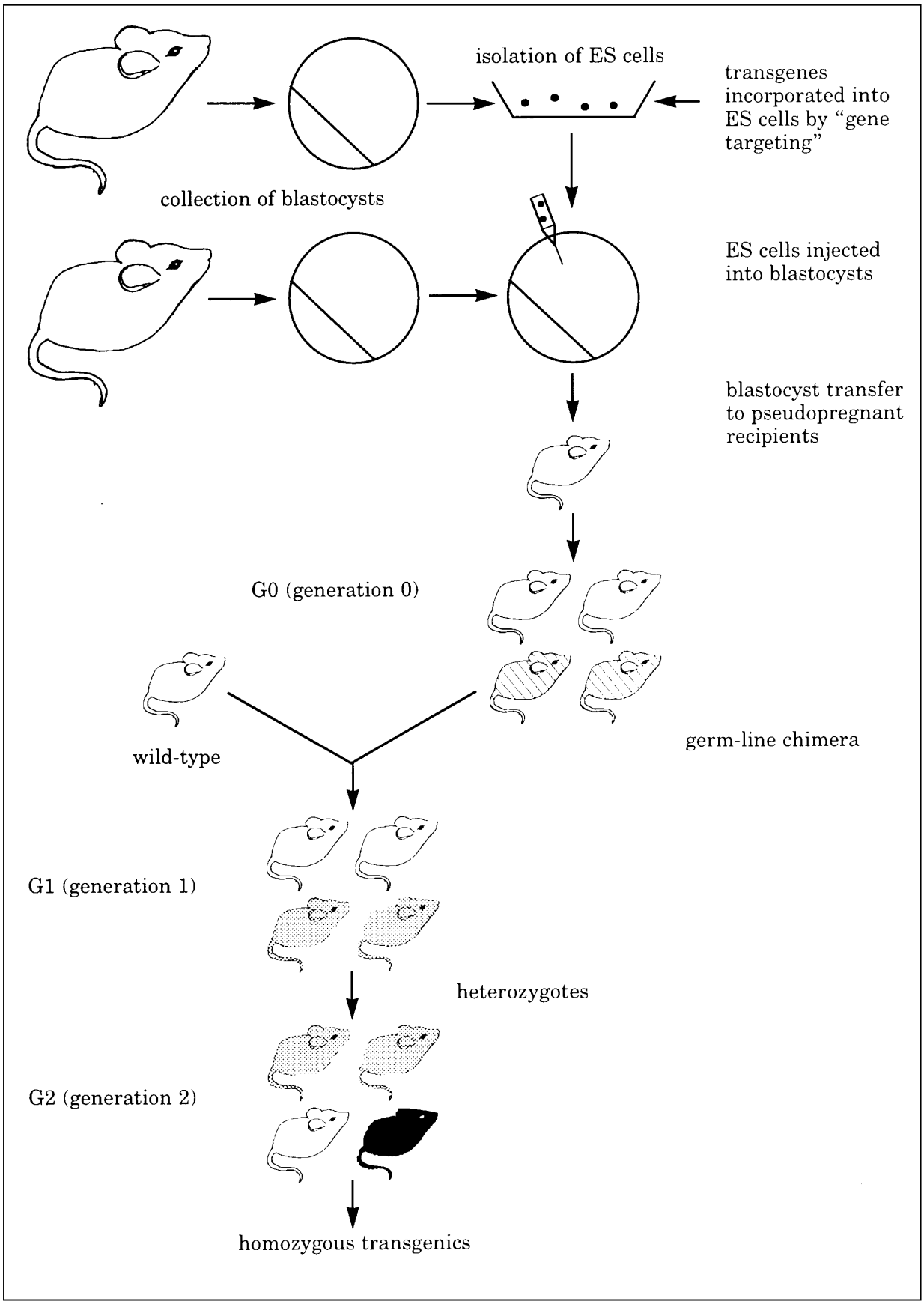

Figure 4: *Embryonic stem cell method (after Mepham et al., 1998)*

ES cell harvesting

Superovulation is induced in normal female mice before they are mated with males. The females are sacrificed approximately 14 to 17 hours after mating (Doetschman, 1994). The abdomen is opened and the embryos are rinsed from the uterus and placed onto a petri dish containing a suitable medium. The ES cells are removed from the embryo and cultured. When stored in the appropriate culture they can be maintained in their undifferentiated state for a long period of time. At this point one of the great advantages of the ES cell technique becomes apparent: the ES cells can be selected *in vitro*, not only for incorporation of the transgene, but also for incorporation at the desired place in the chromosome, by selecting marker sequences included in the transgene (Hubrecht, 1995). Genetic engineering of the ES cells as described above can then be applied. The genetic material is usually inserted into the ES cells by means of electroporation. This method involves exposing the ES cells to an electric current, which disrupts the cell membrane configuration, allowing the DNA to enter the cells (Chu et al., 1987; James and Grosveld, 1987). The ES-cell clone can be screened to see if the genetic modification has succeeded. Homologous recombination will only occur in a minority of the modified ES cells. Chu et al. (1987) estimated that this technique is capable of transforming 1% of the viable cells to stable expression of a transgene. Although this part of the technique is not very efficient, the screening of the ES cells makes that pointless injections, transfers and births are avoided by the knowledge that the ES cells used contain a correctly positioned, functional transgene.

Blastocyst injection procedure

The manipulated ES cells are re-inserted into recipient blastocysts. These are usually taken from a mouse with a different coat colour making detection of the transgene in the offspring easier due to their mixed fur colour. The recipient blastocysts are stabilised by a holding pipette and the injection pipette is brought into the blastocoelic cavity. The ES cells are expelled and aggregate with the cells of the inner cell mass where they can participate in embryonic development.

Embryo transfer and development

The injected blastocysts are surgically transferred into the uterus of pseudopregnant females. These are the foster mothers (FM). After development to term, some of the pups that are born (G_0 generation) will be chimeric. They are developed from two distinct embryonic sources: the unmodified ICM cells of the injected blastocyst and the genetically modified ES cells. The tissues of the chimeric mice that are derived from the modified ES cells will be transgenic.

Normally, the modified ES cells are derived from a mouse strain with a different coat colour than the mouse strain that provided the ICM cells, therefore the chimeric mice will be bi-coloured, which makes it easy to recognise them. Non-chimeric mice will be genetically normal (not containing the transgene in any of their cells), due to the failure of the ES cell component to fully participate in the process of foetal development (Moore and Mepham, 1995).

Efficiency

About 25% of the G_0 progeny is chimeric. Only a number of these will turn out to be germ-line chimeras, meaning they can pass on the transgene to their offspring. Although coat colour is not decisive for the accurate identification of the desired germ-line chimeras, it is generally the case that the higher the ES cell contribution to coat colour, the higher the likelihood of germ-line contribution (Papaioannou and Johnson, 1993). If these mice are mated with normal (wildtype) mice, the G_1 generation may include some animals that are heterozygous for the modified gene. When these heterozygotes are bred with each other, one in four of their offspring will have two copies of the modified gene of interest (homozygotes). In most cases, the heterozygotes will be healthy, because their second, normal, copy of the gene will still function. But homozygous animals which have two copies of the mutated gene may show abnormalities that will help to reveal the normal function of the disrupted gene (OECD, 1998).

Implications for animal welfare

The term animal welfare is rather problematic since there is no commonly accepted definition. Also means of assessing welfare greatly vary (Barnard and Hurst, 1996; Rowan, 1997). In the literature, animal welfare often has a different meaning to different researchers. Some equate welfare with “biological fitness”, claiming that welfare is only reduced if the animal’s ability to survive and reproduce is diminished (Barnett and Hemsworth, 1990). Broom agrees that physical condition is important, and states that the welfare of a badly injured animal is poor, even if sleep or anaesthesia is temporarily preventing the animal from suffering. However, he adds that the animal’s welfare may also be poor in the absence of physical problems, for example if the animal is frightened, anxious, frustrated or bored (Broom, 1988, 1991). To other researchers, the animal’s welfare is considered to be impaired only if it is experiencing an unpleasant (mental) state. Thus, an animal with a tumour from which it does not suffer has no welfare problem (Duncan and Petherick, 1989; Dawkins, 1990; Sandoe and Simonsen, 1992).

The way welfare is defined influences the types of measures used to describe an objective assessment of impaired welfare. Assessing welfare is relatively simple if breeding performance and physical health are the only measures to be used. However, genetic manipulation can bring about many and different effects in an animal. Sensory functioning, structure of bones and muscles, hormone production, detoxification ability, neural control mechanisms and many other aspects can be affected. To assess the consequences of such effects for the welfare of the individual, careful comparisons of modified or treated animals with control animals must be carried out, using a wide range of measurements of welfare (Broom, 1993).

The question remains whether we can expect that the well-being of animals produced by transgenesis is more at risk than of animals produced by traditional breeding methods. It can be argued that there is indeed an increased risk of welfare problems in animals produced by microinjection techniques. The process of transgenesis can cause welfare problems, beyond those that are caused by the effect of the genetic trait that is introduced or changed.

Most transgenic animals are still being produced by the microinjection method. This method of transgenesis can cause effects on the health and welfare of animals at different levels (Van Reenen and Blokhuis, 1993):

- *At the level of the experimental procedures that are necessary to introduce the DNA construct into the zygote*

The various methods and techniques used for the introduction of the DNA construct into the zygote may have implications for animal welfare. Microinjection can cause mechanical damage to the DNA of the embryo. The production of transgenic animals also involves *in vitro* embryo culture and transfer of the altered embryos into female animals to complete their development. Each of these treatments may affect the normal development, and thus the health and welfare, of the individual. The parental animals (donor animals, vasectomised males, and foster mothers) which are used for the production of the transgenic progeny also experience discomfort as a result of the experimental procedures, such as anaesthesia and injections.

- *At the level of integration of the microinjected DNA into the genome*

Most transgenic animals are produced by the microinjection method, in which the transgene is randomly integrated into the host genome, meaning that the transgene(s) may be inserted anywhere within the host chromosomes. Foreign DNA may disrupt the expression of a natural gene and an insertional mutation can occur. If such mutations occur in physiologically important endogenous genes, there could be subsequent adverse effects on animal welfare.

- *At the level of expression of the introduced gene*

Welfare problems can also occur either as a direct result or as a side effect of the expression of the transgene. To what extent the expression of the introduced gene construct leads to detrimental effects depends on the nature of the deviation caused by the transgene.

Preliminary studies

In order to obtain more insight into some of these aspects, a preliminary inventory study has been performed in 1994 at the Department of Laboratory Animal Science (Utrecht University, The Netherlands). This study was limited to the Netherlands and included interviews with researchers producing or using transgenic animals and with animal welfare officers. In addition, a literature survey on applied techniques, research purposes and welfare aspects of transgenic animals was carried out. An attempt was made to differentiate between different causative factors of welfare problems. The results were presented in the report "Transgenese bij dieren" (van der Meer, 1994).

In 1995 the study was extended to the other EU-Member States. Data have been collected as part of a project funded by the European Commission (EC). The project has been carried out within the scope of the research programme on Socio-Economic Impact Studies of Biotechnology of the European Commission (BIOTECH programme DG-XII). The aim of this project was to provide insight into the extent of use of transgenic animals in the EU-Member

States and to reveal information on welfare studies in this area of research. The project also aimed to generate information for the design of a study for the development of a test protocol, which could be used for evaluating welfare problems in the production of transgenic animals (Van der Meer and Van Zutphen, 1997). The methodological approach of the project included a literature survey and an inquiry of the national contact points (NCPs; the governmental representatives for legislative issues on animal protection) of the different EU-countries. As part of the project, a workshop with international participants was organised in order to discuss the welfare aspects of transgenic animals and to disseminate information about the project to other parties. The proceedings of this workshop, containing the papers of the presentations of the invited speakers and the summary of the general discussion, have been published separately (Van Zutphen and Van der Meer, 1997).

From the literature survey, it appeared that research, specifically investigating the effects of transgenesis on the welfare of animals, is rather limited (see Van Reenen and Blokhuis, 1993; Broom, 1993; Moore and Mepham, 1995; Poole, 1995; Hubrecht, 1995).
The information obtained from the NCPs and animal welfare officers confirmed that research aiming at welfare aspects of transgenic animals is scarce. None of the research institutes involved in the production of transgenic animals has carried out specific welfare research with transgenic animals. Some local animal experimentation committees propose monitoring of the transgenic animals, but this is certainly not common practise yet (Cohen and Hazekamp, 2000). In some cases a checklist is used; in other cases the animals are weighed every week. Whenever changes are observed, the welfare officers and the researchers involved are informed.

All institutes involved in the survey mentioned housing of the transgenic mice as a main problem. Transgenic animals must be kept in separate rooms. Whenever several transgenic lines are generated and maintained simultaneously, lack of space can occur. It can take some time to obtain the DNA screening results, which also puts pressure on space in the animal facility. Those who were interviewed considered this a major cause of welfare problems, since overcrowding can reduce growth and increase the chance of infections. The possibility of cryopreservation (freezing) of the transgenic strains has been indicated as an urgent need.

Based on the data collected from both preliminary studies, an overview could be made of the experimental procedures involved in the process of transgenesis that can cause discomfort in parental animals.

Mice are often housed individually during the procedures (especially the vasectomised males). Individual housing of mice is known to cause discomfort (Brain, 1975). The donor mice, providing the fertilised eggs for microinjection, receive one or two hormonal treatments with gonadotrophins, in order to increase the number of eggs released by the ovary. Most of the female mice used for this superovulation are rather young when first mated (at the age of 3-4 weeks), at least before their first ovulation, to ensure the effectiveness of the superovulation hormones. This early mating may involve discomfort (Hubrecht, 1995). The fertilised eggs are collected from the donor mice. Therefore these animals are euthanized 12-16 hours after mating. The foster mothers are submitted to a surgical procedure under general

anaesthesia for the implantation of the microinjected oocytes. With a fine glass needle the embryos are inserted via the infundibulum into the oviduct of the foster mothers. After nursing the progeny, the foster mothers are euthanized as are the non-transgenic offspring.

Male mice used for mating with the foster mothers to induce pseudopregnancy prior to the implantation, are submitted to a surgical procedure under general anaesthesia to be vasectomised (transection of the vas deferens). In contrast to the females who are killed after egg-donation or pregnancy, the males can be used more than once and for a longer period of time.

In ES cell studies there is no need to generate multiple lines of offspring because before insertion, the researcher selects *in vitro* the ES cells with the desired modification. Moreover, because the gene is targeted, the expression of the gene is more standardised. Both of these considerations mean that fewer offspring and hence fewer recipient females are required in ES cell studies (Hubrecht, 1995).

The success rates of the two transgenic techniques are variable and depend on the gene introduced and the mouse strains used. In the process of generating transgenic animals the percentage of eggs that finally develop completely and the percentage of transgenics depend, to a large extent, also on the skill of the technician who carries out the technical procedure and on the quality of the equipment used. With the microinjection technique, approx. 10-30 % of the foster mothers' progeny is transgenic. This rate is increased when the embryonic stem cell technique is used, up to approx. 25-80%, depending on the modified embryonic stem cell clone used (Moore and Mepham, 1995).

At the start of the present project, only limited information was available regarding the potential adverse effects of the manipulations with the oocyte or the embryo for the progeny. Gene transfer by microinjection is not a procedure with a predictable outcome, but rather a trial and error procedure with many unsuccessful attempts and outcomes. It may diminish the viability of the embryos, for instance by mechanical damaging the DNA of the zygote, or because there is usually no control over where the gene inserts itself into the genome of the recipient, or over the number of copies of the gene which become incorporated (Poole, 1995).

When a transgene integrates into the genome, the construct may disturb the expression of the natural genes and an insertional mutation can occur. According to the literature, these unintended mutations occur in 5-15% of the total number of transgenics after microinjection and in < 5% when embryonic stem cells are used (Covarrubias et al., 1986; Constantini et al., 1989; Meisler, 1992). These frequencies may be higher, because some mutations can be lethal at an early stage, while others stay undetected (internal or behavioural) or become manifest only after continued breeding (homozygotes). If such mutations occur in physiologically important endogenous genes, there could be subsequent adverse effects on animal welfare (Rijkers et al., 1994). The degree of discomfort will depend on the function of the mutated gene.

Besides the introduction of the DNA, the production of transgenic animals also involves *in vitro* embryo culture and the implantation of these embryos into the foster mothers. Each of these treatments may affect the normal development and thus the health and

welfare of the animal. However, the discomfort for the progeny caused by these treatments is difficult to establish, because less vital animals may not be born or weak animals may be cannibalised soon after birth by their mother. Because such maternal killing appears to occur more often with transgenic animals than with non-transgenic animals in some cases, this might be indicative of impaired viability as a result of the transgenic technique.

Most apparent welfare problems seem to occur as a direct result or as a side effect of the expression of the transgene. The extent to which the expression of the introduced gene construct may lead to detrimental effects depends on the nature of the deviation caused by the transgene. Substantial discomfort may be expected to occur in the production of transgenic animals for some human disease models or cancer research. This is not essentially different from the discomfort caused in animal models produced according to traditional breeding techniques. However, examples have been described where the expression of the gene has such severe side effects, that the welfare is seriously compromised. This can be illustrated by the so-called “giant mouse”, which has been genetically engineered using a human growth hormone gene (hGH). These mice are not only larger and heavier than normal laboratory mice, but they also show severe side effects, like chronic kidney and liver dysfunction, tumour development, structural changes in heart and spleen, high infant and juvenile mortality, shortened lifespan and reduced fertility (Brem and Wanke, 1988; Berlanga et al., 1993; Wolf and Wanke, 1997). Poole (1995) discussed the ethical and scientific implications of the use of these animals. He indicated that the quality of science and the value of the likely outcome should be balanced against the suffering inflicted on the animal. Since the welfare of the hGH mouse is seriously compromised, Poole questioned whether this transgenic animal can be considered as an appropriate model. Wolf and Wanke (1997) indicated that the pathogenesis of glomerulosclerosis and the mechanisms involved in hepatocarcinogenesis can adequately be studied in hGH transgenic mice. Also, therapeutic strategies can be tested before the animals suffer from the consequences of renal insufficiency. They stated that the well-being of these hGH transgenic mice may be less disturbed than that of respective conventional animals used for studying these problems. The multiple pathologies described above occur when the animal ages, but when using young transgenic animals for this research, the suffering of the animals might be limited.

Another example of a transgenic animal in which the normal development is seriously disrupted is the "legless mouse". A construct, consisting of a fruitfly gene (heat shock protein) and a viral gene (thymidine kinase) has been inserted into mice (McNeish et al., 1988, 1990). The heterozygous mice were normal, but after inbreeding homozygous mice were born with major developmental abnormalities, including a virtual loss of hind limbs, malformed front limbs, facial clefts and massive brain defects. These animals died within 24 hours after birth. The transgene had disturbed the part of the genome, which plays an important role in the development of limbs and brains. Although the effects arose as side effects, they now became subjected to further embryogenetic studies.

Several other examples with various degrees of discomfort, due to direct or indirect effects of the expression of the transgene are described in the literature (Archibald et al., 1990; Janne et al., 1994; Appleby, 1998).

From the preliminary studies, the conclusion was drawn that the degree of suffering caused by the biotechnological procedures (including insertional mutations) is, in general, less severe than the welfare problems caused by the direct effect of the expression of the transgene (Van der Meer 1994; Van der Meer et al., 1996; Van der Meer and Van Zutphen, 1997). However, it was recognised that only very few data are available to substantiate this conclusion and that, in order to gain more insight into the potential welfare problems caused by transgenesis, further research is necessary to identify and quantify indicators of discomfort. Therefore, the object of further research should be to differentiate effects of biotechnological procedures from effects caused by the expressed transgene and to select potential indicators of discomfort that can be monitored on a routine basis. These indicators need to be sensitive, non-invasive and easy to determine (Van der Meer and Van Zutphen, 1997).

Scope of the thesis

The mouse is by far the most frequently used animal species in transgenesis. So far it is not clear whether the transgenic technique as such influences the well-being of transgenic mice. To obtain more insight into this aspect, the (harmful) effects of the biotechnological procedures must be differentiated from the (harmful) effect of the expressed or mutated transgene. The research described in this thesis aims at studying the effects of the biotechnological procedures involved in genetic modification by applying two different transgenic mouse techniques, one using the microinjection technique, the other using gene targeting of embryonic stem cells. A test protocol was developed for the systematic observation of the animals and for collecting information that might be relevant for establishing the impact of transgenesis on the well-being of these animals (Van der Meer and Van Zutphen, 1997). Within each of the transgenic experiments the physiological and ethological parameters included in the protocol, are screened in different groups of animals, all with a different transgenic background. By this approach the effect of the technique can be differentiated from the effect caused by the expression or knockout of the transgene. Besides testing whether the biotechnological procedures of transgenesis as such have an influence on the well-being of animals, this thesis also aims to evaluate the feasibility of the test protocol for monitoring welfare aspects. Based on a critical evaluation of the welfare parameters used in the test protocol, score sheets have been developed for the routine control of welfare problems in the production of (transgenic) mice.

The first part of the study emphasised the search for differences in the early postnatal development between three experimental groups, each with a different transgenic background, produced with the microinjection technique, and a control group. To this end, newborn mice were subjected to various behavioural tests and the growth and development of their morphological characteristics were recorded from birth until weaning (Chapter 2). Chapter 3 describes the monitoring of the same groups of mice in their post-weaning period (4-30 weeks of age), in order to compare different aspects of behaviour. Also their morphological and physiological development was monitored up until the age of 30 weeks, after which post mortem examinations were performed.

In Chapters 4 and 5, the effects of gene targeting procedures have been investigated. The same parameters were used for testing as have been described for the microinjection technique. Chapter 4 describes the ontogenesis of behaviour and morphology from birth until weaning of different experimental groups, while in Chapter 5 the post-weaning period of these groups is described. Subsequently, a scoring system has been developed, containing a limited number of sensitive, easy to determine and non-invasive parameters, selected from the previous studies on implications of transgenesis for the well-being of mice. The feasibility of this scoring system has been tested, to assess the use of score sheets for monitoring the welfare of transgenic mice on a practical basis, as part of the animal technicians' daily routine in a transgenic unit (Chapter 6). Finally, in Chapter 7 the results of the experiments described in this thesis are evaluated and practical implications for animal welfare and animal experimentation are discussed.

Chapter 2

Welfare assessment of transgenic animals: Behavioural responses and morphological development of newborn mice

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Summary

Four groups of mice of the same inbred strain, but with different transgenic backgrounds (no treatment; integration of a functional corticotropin-releasing factor (CRF) gene construct; integration of a non-functional CRF gene construct; transgenic technique without integration of a DNA construct) were compared, in order to identify and quantify indicators of discomfort in transgenic animals. This approach enables us to differentiate between the effects of the technique of transgenesis and the effects caused by the expression of the transgene. This chapter emphasises the search for differences in the early postnatal development of the animals. To this end, newborn mice have been subjected to various behavioural tests; moreover, their growth and morphological characteristics were measured from birth up to the age of 3 weeks. The results indicate that the presence of the microinjected DNA-construct influences the survival rate during the first 2-3 days after birth. 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. The increase in the body weight of pups with a functional CRF construct was significantly lower than in the other groups, but only during the first 11 days. No significant differences in morphological characteristics or behavioural development were observed between the four groups. This approach was found to be adequate for detecting a broad variety of behavioural and morphological characteristics. Before general conclusions about the extent to which the technique of transgenesis affects animal welfare can be drawn, more transgenic lines should be studied in this way.

Introduction

Since the first transgenic mice were generated in 1980 (Gordon et al., 1980; Gordon and Ruddle, 1981), transgenesis has become a widely used technique, involving an increasing number of animals. Although the number of published papers on animal welfare has also increased substantially in recent years, only very few studies have been published on evaluating the welfare aspects of transgenic animals (Van Reenen and Blokhuis, 1993, 1997; Broom, 1993; Moore and Mepham, 1995; Poole, 1995; Hubrecht, 1995; Van Zutphen and Van der Meer, 1997). In fact, it remains to be proven whether the welfare of animals produced by transgenesis is more at risk than the welfare of animals produced by traditional breeding methods, or, in other words, whether the technique of transgenesis is, in itself, a threat to animal welfare.

The majority of transgenic animals are produced by the pronuclear microinjection method. The injected transgene is randomly integrated into the recipient genome and may cause a change in the normal physiological processes of the animal. The consequences for the welfare, including the health, of transgenic animals not only depend on the expression of the gene construct (Van Reenen and Blokhuis, 1993; Moore and Mepham, 1995). Manipulations of oocytes or embryos, or the disruption of parental DNA at the integration site of the gene construct (insertional mutations) can also influence the normal development of the transgenic animal. Moreover, the parental animals (superovulated females, vasectomised males, foster mothers) used for the production of the transgenic progeny can also experience discomfort from the experimental procedures to which they are exposed. Based on present knowledge, it is not possible to judge whether the transgenic technique itself causes the resulting offspring any discomfort.

Four groups of mice of the same inbred strain, but with various transgenic backgrounds (no transgenic treatment; integration of a functional corticotropin-releasing factor (CRF) gene construct; integration of a non-functional CRF gene construct; transgenic technique without integration of a DNA construct) were compared, in order to identify and quantify indicators of welfare in transgenic animals. By using this approach, the effects of the technique can be differentiated from the effects caused by the expression of the transgene. A test protocol was developed for the systematic observation of the animals and for collecting information which might be relevant for establishing the impact of transgenesis on the welfare of these animals (Van der Meer and Van Zutphen, 1997). Mice produced in current transgenic experiments are being observed from birth up to 30 weeks of age, and screened for a number of physiological/clinical and ethological parameters which are frequently used in studies to assess discomfort. These parameters include survival rate, growth, reproductive capability, and various morphological, clinical and behavioural characteristics (Broom, 1991).

In particular, the ontogenesis of behaviour can be considered to be a sensitive indicator of discomfort. The use of behavioural tests to study functional and behavioural changes in offspring can therefore be seen as an important tool in the assessment of potential discomfort in genetically modified animals (Costa, 1997a). Screening the behaviour of transgenic mice

from birth will permit eventual deficits to be identified, estimated and quantified. Besides testing whether the technique of transgenesis itself can influence the welfare of animals, this project is also aimed at evaluating the feasibility of including various parameters in a test protocol for monitoring welfare aspects on a routine basis in a transgenic animal facility.

This paper deals mainly with the use of behavioural tests and screening of the morphometric development of newborn mice as possible tools for establishing the effects of the technique of transgenesis. The transgenic mice were selected from a project currently running in the Department of Psychopharmacology, Utrecht University. CRF, a hypothalamic peptide, is secreted in response to various stressors. One of its actions is to induce adrenocorticotropic hormone (ACTH) release from the pituitary gland, leading to increased adrenal corticosteroid production. Changes in CRF expression may have important physiological consequences. CRF transgenic mice were produced as a model for chronic stress (chronic fear and/or depression), with the aim of investigating the role of CRF in the development and pathogenesis of anxiogenic behaviour. The CRF gene has been introduced by microinjection in C57BL/6 mice, and the resulting transgenic mice show signs of chronic pituitary activation, decreased growth, increased basal serum levels of corticosteroids, and features of Cushing's syndrome, such as hair loss and thinning skin.

Animals and Methods

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 postnatal development (0-3 weeks), and differed only in their transgenic backgrounds.

Group 1: CRF transgenic animals

This group consisted of two subgroups of 25 (Group 1a, 9 females and 16 males) and 23 (Group 1b, 13 females and 10 males) transgenic mice. Each subgroup was derived from a different CRF transgenic founder male, which was crossed with a wildtype 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 fertilised 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 (PCR) with transgene-specific primers.

Group 2: non-functional CRF transgenic animals

This group consisted of two subgroups of 13 (Group 2a, 8 females and 5 males) and 20 (Group 2b, 7 females and 13 males) transgenic mice with a non-functional CRF construct.

In the non-functional 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 fertilised eggs (C57BL/6N Crl), and the same procedure was performed as described for Group 1; each subgroup was derived from a different founder male (non-functional CRF construct), which was crossed with a wildtype C57BL/6N Crl female.

Group 3: non-transgenic animals after transgenic procedures
This group consisted of two subgroups of 26 (Group 3a, 11 females and 15 males), and 7 (Group 3b, 3 females and 4 males) 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 which were manipulated in the same way, but without a gene construct in the injection needle (the male pronucleus was penetrated, but nothing was injected).

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) were tested.

Housing and handling conditions
The pregnant C57BL/6N Crl mice were individually housed in wire-topped Macrolon type II cages (410 cm^2; UNO Roestvaststaal, Zevenaar, The Netherlands) with sawdust bedding (Pine-wood ¾; Woodyclean, BMI, Helmond, The Netherlands). A tissue (Kleenex, Kimberley-Clark, Ede, The Netherlands) was added to each cage for nest-building. The foster mothers were individually housed after the implantation procedure. Animals were housed conventionally and maintained under standard conditions (12 hours light: 12 hours dark cycle with lights on from 0600 hours to 1800 hours, 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*.

On the day they were born, the pups were weighed and inspected for any malformations or special traits. Each pup was then marked with a blue dye (Pentel felt pen F50, permanent marker) on the skin. The marks were renewed daily during the pre-weaning period to maintain their identification. From day 10 until day 21 (with the onset of hair growth), picric acid was used for identification (under the armpits). This had to be renewed every 4 to 5 days. For each litter, the number of dead pups was recorded daily from day 0 until day 21. During the first 5 days the pups were inspected for the presence of the milk spot (a white spot in the stomach) as an indicator of milk uptake.

Pups from different litters were handled with clean gloves. During the tests, the mother was placed in a separate cage at the other side of the room. To prevent the pups from cooling down, they were kept on a heating pad. Before replacing the pups in their home cages, they were rubbed softly with dirty bedding material from this cage, to prevent rejection by

their mother. Stress among the mothers, resulting in cannibalism of young pups, occurs especially during the first 4 days after birth. Therefore, cleaning of the cages was avoided just before, during or after parturition, and nesting material (paper tissues) was added.
Mice were weaned at the age of 3-4 weeks. The pups were weighed daily (days 0-21), and thereafter were subjected to a set of behavioural tests and morphological screenings.

Behavioural tests
A modified version of the test battery developed by Fox (1965) and Altman and Sudarshan (1975) was used to screen for possible behavioural abnormalities in each of the categories of animals (Costa, 1997b). Four levels of response to these tests could be distinguished: 0 (behaviour or response is absent), 1 (signs of primitive response), 2 (a clear but not yet mature response) or 3 (a mature and full response in all aspects of execution such as co-ordination or strength). The animals were subjected to the following tests:

Surface righting (labyrinth and postural response)
The test was performed by placing the pups on their backs with their limbs pointing upwards. The maximum response was to turn over immediately and stand on all four limbs.

- Level 0: no response;
- Level 1: the animal lies on its back, moving its paws in the air without any direction, no righting yet;
- Level 2: the animal rights itself, but slowly; the direction of the paws is more controlled in the righting direction;
- Level 3: the animal rights itself immediately and stands on its four paws.

Walking (co-ordination and muscular strength)
This includes the testing of the development of locomotion.

- Level 0: no locomotion;
- Level 1: pivoting - moving around with the help of the head and forelimbs, but not using the hind limbs;
- Level 2: crawling - moving on all four limbs, dragging the belly over the surface;
- Level 3: walking - mature locomotion with the body supported completely by the four limbs.

Cliff drop aversion test (somato sensory response)
The pups were individually placed on the edge of a surface of polystyrene foam (height 3cm) with the forelimbs and front of the head partially off the foam. The mature response is that the animal quickly turns its head and forelimbs and avoids dropping.

- Level 0: animal falls off the foam;
- Level 1: the animal turns very slowly back to the surface;
- Level 2: the animal avoids the cliff, but it still takes some time to turn;
- Level 3: the animal immediately turns back.

Geotaxis test (labyrinth and postural reaction)

The pups were placed in a head-down position on a surface of polystyrene foam, which was inclined at a slope of 45°. The mature response is when the animal immediately rotates 180° to the head-up position and starts to climb up the slope.

- Level 0: the animal does not move at all;
- Level 1: the animal turns its body slowly up the slope, but stops halfway without moving any further;
- Level 2: the animal turns its body, stops halfway and then continues turning up to 180°;
- Level 3: the animal turns immediately by 180° and moves towards the top of the slope.

Grasping test (freeing reflex)

The palm area of one of the forelimbs is stimulated with a thin cotton bud. The mature response is when the animal immediately grasps it. This reflex disappears with the development of the nervous system.

- Level 0: no grasping;
- Level 1: the animal puts its paw on the cotton bud, but it does not hold on firmly;
- Level 2: the animal puts its paw on the cotton bud with more force, but when the cotton bud is pulled, cannot hold it;
- Level 3: the animal grasps the cotton bud very firmly.

Bar holding test (muscular strength)

The pup has to grasp a bar (cotton bud) and its ability to hang on to the bar is observed. The mature response is when the animal holds the bar with all four limbs, with its tail wound around the bar.

- Level 0: animal cannot hang on to the bar;
- Level 1: animal holds on to the bar with forepaws only;
- Level 2: animal can put its hind paws onto the bar, but not yet its tail;
- Level 3: animal puts all its four paws and tail around the bar.

Morphological screenings

Morphological development, such as the age of onset of incisor eruption, nipple development, eye-opening, ear-elevation and opening, and hair growth, was determined by daily observation of the individual animals (days 0-21).

Hair growth

- Level 0: no hair present, animals are still bald;
- Level 1: fur starts as fine stubble over the back;
- Level 2: complete coat of fine fuzzy fur is visible;
- Level 3: animals are totally covered with thick hair.

Incisor eruption (lower and upper incisor)

- Level 1: incisors are visible, but not erupted;
- Level 3: incisors are erupted.

Eye opening

- Level 1: eyelids start to open, slit-like palpebral opening;
- Level 3: eyelids are totally open, oval palpebral opening.

Ear elevation

The ears of newborn pups lie flat against the head. A level of 3 is given when both ears are elevated 45° away from the head.

Ear opening

- Level 1: ears start to open, slit-like opening;
- Level 3: ears are totally open.

Statistical analysis

All data from the various observations were statistically analysed (by using SPSS 8.0 as statistical package), to determine whether there were any significant differences between the treatment groups and the control group. For the behavioural and morphological parameters, data were evaluated by using non-parametric statistics, i.e. the Kruskal-Wallis test, and, where appropriate, the Mann-Whitney *U* test with the Bonferroni correction. The level of statistical significance was pre-set at $P \leq 0.05$ for all the parameters. Since the body weight of the pups during the pre-weaning period consisted of repeated measurements, data were assessed with longitudinal analysis (by using S Plus 4.0). Different models were compared using Likelihood Ratio tests.

For the statistical analysis, data were used from all the pups that survived day 2. Statistical analyses were performed, using the litter as the unit of measurement, by computing the mean value of data for the pups in each litter. As Group 3b consisted of only one litter, the data from this group were not included in the statistical analysis.

Results

Survival rate

In Groups 1a and 1b, four pups died before day 2 (Table I). This loss occurred in two litters; thus, in four out of the six litters of these groups, no pre-weaning loss occurred. In Group 2a, nine pups died before day 2. This loss occurred in four litters; thus in two out of six litters, no pre-weaning loss occurred. In Group 2b, one pup died before day 2. In Groups 3 and 4, all pups that were born survived the pre-weaning period. There was no significant difference in litter size between the treatment groups and the control group at birth (Table I).

The numbers of animals available for testing were 25 (16 males, 9 females), 23 (10 males, 13 females), 13 (5 males, 8 females), 20 (13 males, 7 females), 26 (15 males, 11 females), 7 (4 males, 3 females) and 26 (9 males, 17 females) for Groups 1a, 1b, 2a, 2b, 3a, 3b and 4, respectively.

Table I: *Survival rates of mouse litters in the four test groups [a] (day 0-21)*

Parameter	Group 1a	Group 1b	Group 2a	Group 2b	Group 3a	Group 3b	Group 4
No. of litters	6	6	6	6	7	1	4
No. of offspring	44	44	44	43	28	7	26
Litter size [b)]	7.3±0.52	7.3±2.43	7.3±1.86	7.2±2.04	4.0±1.53	7.0±0.00	6.5±0.58
Pups dead [c)]	4	4	9	1	0	0	0
Transgenics [d)]	62.5%	57.5%	37.1%	47.6%	--	--	--

[a)] Group 1: Corticotropin-releasing factor (CRF) transgenics with functional CRF construct (1a and 1b represent progeny from two different founders); Group 2: CRF transgenics with non-functional CRF construct (2a and 2b represent progeny from two different founders); Group 3: non-transgenics after transgenesis (3a, injected with DNA construct, no integration; 3b, transgenic procedure, but no construct injected); Group 4: control animals (no transgenic treatment).
[b)] Mean ± SD.
[c)] Pups all died on day 0, day 1 or day 2. Dead pups were not tested for transgenicity.
[d)] Based on pups alive after day 2.

Weight curve

The birth weights of mice in Groups 1, 2 and 3 were not statistically different from those in the control group (Figure 1). Analysis of the development of body weight during the first 11 days of the pre-weaning period indicated a significantly reduced average weight for Groups 1a and 1b, when compared to the control group (Group 4). Within Groups 1a and 1b, the transgenic pups also showed reduced weights compared with their non-transgenic litter-mates (data not shown). Pups in Groups 3a and 3b showed a significantly increased weight compared to Group 4. For Group 3a, this might have been due to the smaller litter sizes. There was no significant difference between the weights of the pups in Group 2 and those in the control group. The differences in growth rates between Groups 1 and 3 and the control group no longer existed after 11 days.

The maximum period (in days) before a certain behaviour is fully developed in control C57BL/6N Crl mice is showed in Figure 2. This period is the time between the first observation of levels 1 and 3.

Behavioural observations

A summary of the results of the behavioural tests is given in Table II. Statistical analysis revealed a significant difference only in the cliff drop aversion test for Groups 1b, 2a and 2b (level 1), in which animals started the response sooner than the animals in the control group. The pups in Group 3b also started sooner than those in the control group, but as Group 3b

consisted of only one litter, the data from this group were not included in the analysis. No other statistically significant difference was found between the treatment groups and the control group in any of the behavioural tests employed.

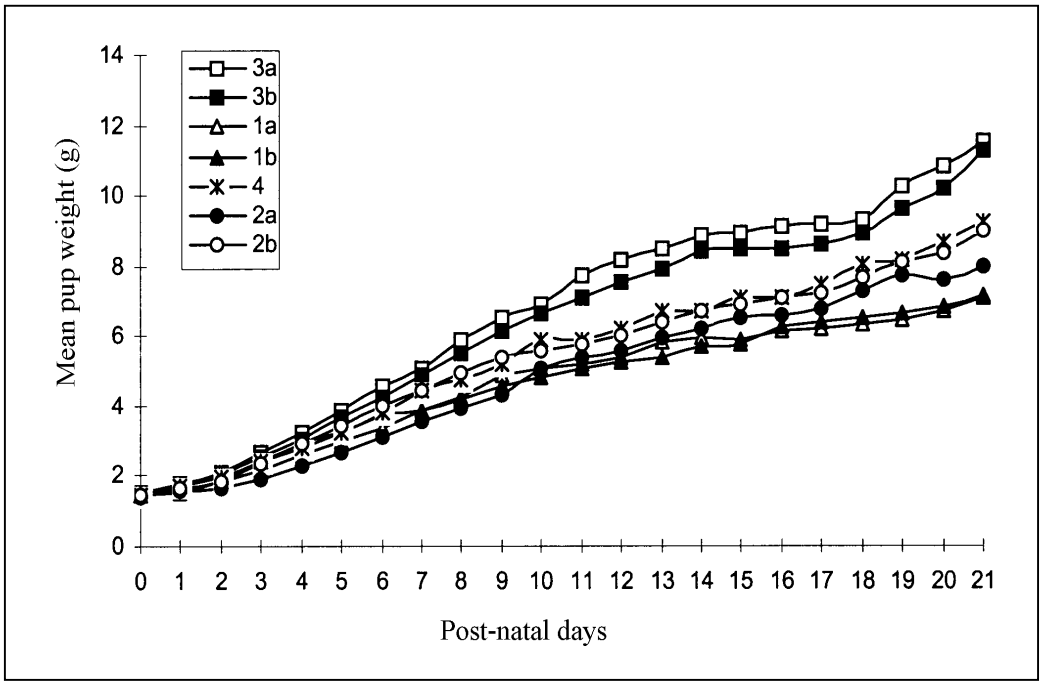

Figure 1: *Mean pup weight (g) in pre-weaning period.*
All pups of the different groups were weighed daily from day 0 until day 21.

Morphological development

Table III shows the normal development of external characteristics in control mice (C57BL/6N Crl) from birth until 3 weeks of age.

A summary of the development of morphological characteristics in the various groups is given in Table IV. In Group 2a, hair growth started at a later age, whereas, in Group 3, hair growth started earlier than in the control animals. Starting from day 11, the pups of the two transgenic lines with the functional CRF construct (Groups 1a and 1b) showed a different pattern in hair growth when compared to the other mice. The fur became thinner (skin was visible), starting on the head and neck and spreading to the rest of the body. They also develop a differently shaped head, which was wider and shorter than that of normal mice. No significant difference in onset of the development of hair growth was observed in Groups 1a and 1b compared to the control group.

The external ear of the newborn mouse is small and adheres to its head. On day 3 in wildtype C57BL/6N Crl mice, the ears are elevated about 45° away from the head, while at day 4, this is 90°. At day 11-13 the ears are open. The eyelids of normal pups open from day 13 to day 15, first as a slit-like palpebral opening (level 1), and finally as an oval palpebral opening (level 3).

***Table II**: Summary of behavioural tests*

Test	Group 1a	Group 1b	Group 2a	Group 2b	Group 3a	Group 3b	Group 4
Walking							
Level 1	2.68±0.63	2.61±0.50	2.92±0.28	3.00±0.00	2.62±0.50	3.00±0.00	2.57±0.50
Level 2	5.48±0.65	4.91±0.85	6.23±0.60	5.70±0.47	6.31±0.79	5.57±0.79	5.31±0.74
Level 3	8.68±0.69	8.17±1.03	8.31±0.48	8.85±1.14	8.38±0.64	8.29±0.49	8.65±0.94
Period	6.00±0.87	5.57±1.16	5.38±0.51	5.85±1.14	5.77±0.82	5.29±0.49	6.08±0.98
Cliff drop							
Level 1	3.35±0.71	2.78±0.42*	3.00±0.00*	3.00±0.00*	3.19±0.40	3.00±0.00	3.31±0.47
Level 2	5.39±0.72	4.70±0.63	5.31±0.48	5.15±0.37	5.27±0.67	5.00±0.00	4.92±0.63
Level 3	8.04±0.91	7.61±0.66	8.08±0.49	7.65±0.59	8.08±0.69	8.00±0.00	7.54±0.65
Period	4.65±1.15	4.83±0.83	5.08±0.49	4.65±0.59	4.88±0.95	5.00±0.00	4.23±0.76
Geotaxis							
Level 1	3.39±0.72	2.83±0.39	3.00±0.00	3.10±0.45	3.15±0.46	3.00±0.00	3.31±0.55
Level 2	5.65±0.65	5.17±0.78	5.54±0.78	5.45±0.51	6.00±1.10	5.71±0.95	5.00±0.75
Level 3	8.72±0.79	8.30±0.70	8.92±0.49	8.95±0.60	8.73±0.67	8.86±0.69	8.62±0.75
Period	5.30±1.11	5.48±0.79	5.92±0.49	5.85±0.75	5.58±0.76	5.86±0.69	5.31±0.93
Righting							
Level 1	3.83±0.48	3.00±0.00	3.23±0.44	3.10±0.45	3.12±0.33	3.00±0.00	3.46±0.71
Level 2	5.63±0.58	5.26±0.54	5.77±0.60	5.85±0.67	5.54±1.03	5.57±0.53	5.50±0.71
Level 3	8.17±0.87	8.00±0.85	7.92±0.28	8.30±0.73	8.35±0.56	8.14±0.38	8.12±0.59
Period	4.33±0.76	5.00±0.85	4.69±0.48	5.20±0.89	5.23±0.71	5.14±0.38	4.65±0.94
Grasping							
Level 1	8.18±0.91	7.90±0.91	8.36±0.50	8.00±0.56	8.50±0.65	8.00±0.00	8.31±0.84
Level 2	9.82±0.85	9.45±1.19	10.09±0.83	9.35±0.67	10.12±1.03	9.00±0.00	9.92±1.16
Level 3	12.09±1.02	11.45±1.00	12.18±0.87	10.80±0.89	11.77±1.21	11.00±0.58	11.31±1.23
Period	3.91±0.81	3.55±0.83	3.82±0.75	2.80±0.62	3.27±0.92	3.00±0.58	3.00±0.80
Bar holding							
Level 1	10.84±0.85	10.30±1.30	9.91±0.83	10.05±0.69	9.81±0.69	9.57±0.53	10.38±1.10
Level 2	13.00±0.71	12.50±0.95	12.73±1.19	12.65±0.49	12.27±0.92	12.29±0.76	12.58±0.70
Level 3	16.16±0.75	15.25±0.64	14.91±0.94	15.80±0.41	15.54±1.27	15.14±0.69	15.23±0.65
Period	5.32±1.25	4.95±1.36	5.00±0.77	5.75±0.55	5.73±1.34	5.57±0.79	4.85±1.38

*For each test, the first day of occurrence of levels 1, 2 and 3 are shown. Values are means of all pups per group ± SD. The period (difference between level 3 and level 1) shows the time in days that the animals needed to develop a certain behaviour. * = Significantly different from controls: P≤0.05*

No significant differences in the development of ear-opening or eye-opening were found between the treatment and control groups. Also, there were no differences in the time of onset of the eruption of incisors, with the exception of Group 2, where the eruption of the lower incisors started slightly earlier and the upper incisors erupted somewhat later than in controls. In Group 2a, the period of eruption of the upper incisors was slightly shorter than for the controls.

In control C57BL/6N Crl mice, inguinal nipples appeared in females at day 8 or 9. Only in Group 2b were the inguinal nipples visible at a younger age than was observed in the controls.

Milk uptake is visible in the stomach from birth until day 4 or day 5, when the skin is still transparent (level 3). All pups showed first signs of milk uptake at birth; no significant differences in the appearance of milk spots were found between the treatment groups and the control group.

***Table III**: Morphological development of control C57Bl/6N Crl mouse pups*

Birth	Skin colour blood red.
Day 1	Skin colour lighter. Milk visible in the stomach.
Day 2	Skin colour pink. Ears flat against the head.
Day 3	Ears elevated about 45° away from the head.
Day 4	Ears elevated 90° away from the head.
Day 5	Skin thicker. Milk no longer visible in the stomach.
Day 6	Fur starts as a fine stubble over the back.
Day 7	Complete coat of fine fuzzy fur is visible.
Day 8	Lower incisors visible, but not erupted.
Day 9	Inguinal nipples visible in females.
Day 10	Lower incisors erupted.
Day 11	Upper incisors erupted, fine soft fur.
Day 11-13	Ears open.
Day 13-15	Eyelids open. Slit-like palpebral opening.
Day 21	Oval palpebral opening, triangular shape to head.

The design of this table is based on Table 18.2 of the UFAW Handbook on the Care and Management of Laboratory Animals (Baumans, 1999)

Discussion

The data presented represent the first results of a large-scale study of the impact of transgenesis on the welfare of animals. The criteria used to assess the welfare of animals included survival rate, morphological development, behavioural response, life-span, growth rate, reproduction and post-mortem pathology. The assessment of these parameters contributes to the definition of newly produced animal models, and to the refinement of the housing conditions and breeding protocols, in line with the animals' needs (Costa, 1997a).

***Table IV**: Summary of morphological development*

Morphology	Group 1a	Group 1b	Group 2a	Group 2b	Group 3a	Group 3b	Group 4
Hair growth							
Level 1	7.30±0.70	7.30±0.47	8.27±0.47*	7.50±0.61	6.04±0.20*	6.00±0.00	7.32±0.48
Level 2	8.65±1.15	8.30±0.47	9.27±0.47*	8.55±0.60	7.08±0.27*	7.00±0.00	8.36±0.49
Level 3	10.09±1.35	10.05±0.76	10.45±0.69	9.55±0.60	9.08±0.93	10.00±0.00	9.96±0.89
Period	2.78±0.80	2.75±0.55	2.18±0.40	2.05±0.22	3.04±0.96	4.00±0.00	2.64±0.64
Ears elevated							
Level 3	3.94±0.56	3.10±0.64	3.57±0.53	3.82±0.40	3.20±0.41	3.43±0.53	3.55±0.51
Ears open							
Level 1	12.27±0.55	11.95±0.89	13.09±0.30	12.78±0.43	12.35±0.56	13.00±0.00	12.40±0.96
Level 3	13.36±0.58	13.05±0.89	14.09±0.30	13.78±0.43	13.38±0.64	14.29±0.49	13.40±0.96
Period	1.09±0.29	1.10±0.31	1.00±0.00	1.00±0.00	1.04±0.20	1.29±0.49	1.00±0.00
Eyes open							
Level 1	12.73±0.46	12.35±0.99	13.00±0.45	13.39±0.61	12.69±0.56	13.00±0.00	12.96±0.89
Level 3	14.55±1.10	14.45±1.05	14.27±0.47	14.39±0.61	13.88±0.61	14.29±0.49	14.08±0.81
Period	1.82±1.22	2.10±1.21	1.27±0.47	1.00±0.00	1.19±0.40	1.29±0.49	1.12±0.33
Lower incisor							
Level 1	8.20±0.52	8.62±0.50	8.00±0.00*	8.00±0.00*	8.35±0.49	8.00±0.00	8.36±0.49
Level 3	9.25±0.64	9.62±0.50	9.38±0.51	9.18±0.39	9.70±0.47	10.14±0.38	9.36±0.49
Period	1.05±0.22	1.00±0.00	1.38±0.51	1.18±0.39	1.35±0.49	2.14±0.38	1.00±0.00
Upper incisor							
Level 1	9.86±0.89	10.14±0.65	11.00±0.10*	10.94±0.56	10.85±0.37	11.00±0.00	10.50±0.71
Level 3	11.64±0.79	11.33±0.73	12.08±0.28	12.35±0.61	12.90±0.31	13.00±0.00	11.92±0.74
Period	1.77±0.43	1.19±0.51	1.08±0.28*	1.38±0.50	2.05±0.22	2.00±0.00	1.42±0.50
Nipples							
Level 3	9.00±0.00	9.00±0.00	8.33±0.52	7.00±0.00	8.27±1.19	8.00±0.00	8.65±0.79
Milk spot							
Level 3	3.95±0.38	4.22±0.52	4.00±0.00	4.00±0.00	3.81±0.57	4.00±0.00	4.08±0.65

*For each development, the first day of occurrence of levels 1, 2 and/or 3 are shown. Values are means of all pups per group ± SD. The period (difference between level 3 and level 1) shows the time in days that the animals needed to accomplish a certain development. *=Significantly different from controls: P≤0.05*

By using the parameters described in this study, the impact of the transgenic technique on the early development of animals can be evaluated. Potential indicators of discomfort that can be monitored on a routine basis (for example, by scientists and animal technicians) in the process of producing transgenic animals can be selected.

To study morphological and behavioural development during the pre-weaning period of mice, it is necessary to be able to identify the pups within each litter. There are several identification methods, such as toe clipping, tattooing, ear-marking or colour-marking. We preferred to use a non-invasive method and therefore used a blue marker (Pentel Felt pen F50) on the skin of the mice for the first 10 days. The pups were re-marked daily to maintain their identification. From day 10 (with the onset of hair growth), picric acid was applied under the armpits. This had to be renewed every 4 or 5 days and was found to be an effective way of marking the pups.

In the present study, some adverse effects of the transgenic treatments on survival rate were found. While there were no pup losses scored in Groups 3 and 4, both transgenic lines with a successful expression of the CRF gene, as well as the transgenic lines with the non-functional CRF gene, showed postnatal death of several pups. It should be stated that, in Group 2a, some of the cages were accidentally cleaned during parturition, which may (partly) have been the cause of the high postnatal mortality in this group.

No significant differences in litter size could be detected between the treatment groups and the control group. The average body weight of the pups was lower in Groups 1a and 1b, while it was higher in Groups 3a and 3b compared to the control group.

In rodents, cliff avoidance, surface righting, and negative geotaxis are aspects of behaviour which develop soon after birth (Altman and Sudarshan, 1975; Adams, 1987). The time of their appearance and their development have been suggested as parameters for the assessment of the development of the rodent nervous system and as indicators for the subsequent appearance of more-complex behaviours. The neuromotor development of the young mice, as assessed in this study by applying different tests during early postnatal life, revealed only minor effects of the different transgenic treatments.

Differences in morphological development were found between the transgenic CRF pups in Groups 1a and 1b and the control group. The transgenic CRF mice showed thinner hair and hair loss, and a different shaped head (wider and shorter than wildtype mice), which is probably due to the expression of the CRF gene, because it was not observed in the appropriate controls. Several of these features appear to have been due, in part, to increased corticosterone levels in these animals.

Adrenalectomised transgenic CRF animals revert to a normal phenotype with respect to hair and skin changes within a month after adrenalectomy (Stenzel-Poore et al., 1992; Boehme et al., 1997). The other groups showed only minor differences in morphological development compared to the control group.

Conclusions

In conclusion, the results of this study indicate that there were only a few significant differences between the groups with different transgenic backgrounds and the control group. The integration of a new transgene into the genome without expression of the gene, or procedures employed both before and after microinjection (*in vitro* culture, embryo transfer), seemed to have a limited effect on mice during their first 3 weeks of life, in this particular case.

Although only a few significant differences were found with the behavioural tests, it should be stressed that these tests can be very useful for studying the early effects of transgene expression in newly generated transgenic mice. The tests can be used as a routine procedure in the breeding facility, as they are easy to perform, take little time and are non-invasive. Conclusive evidence of whether, and, if so, when, the procedures of transgenesis jeopardise the welfare of the animals involved will require conscientious monitoring of more animals produced in the development of various transgenic lines.

In the next part of this project, the groups of mice monitored in this study will be subjected to other behavioural tests, in order to compare different aspects of behaviour, such as locomotor activity, anxiety and exploration of an unfamiliar environment. Their morphological and physiological development will be further monitored up to the age of 30 weeks.

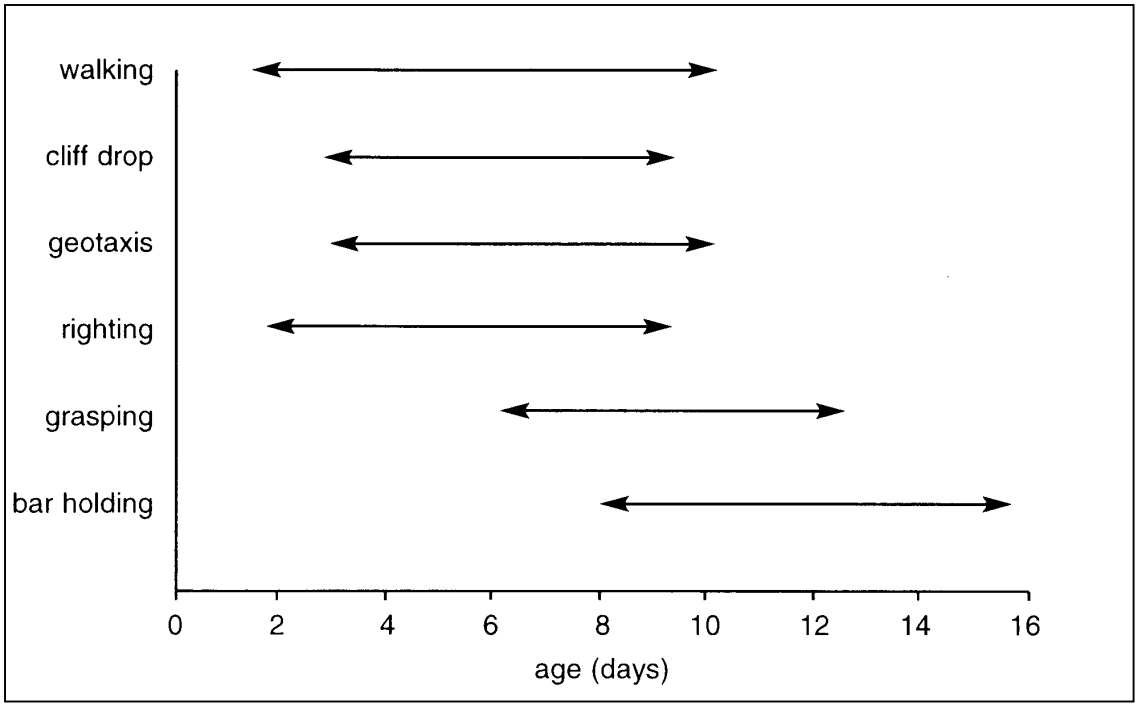

Figure 2: *An overview of the development of behavioural responses in the normal control C57BL/6N Crl mouse pup*

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Chapter 3

Impact of transgenic procedures on behavioural and physiological responses in post-weaning mice

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Summary

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 non-functional CRF gene construct, non-transgenics after transgenic procedures, and controls). These four groups have been tested in various behavioural 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 behaviour, weight gain, and morphology were found between Group 1 (transgenic CRF animals) and Group 4 (control animals). For Group 2 (animals with a non-coding construct) and Group 3 (non-transgenic 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, embryo transfer) have no significant effect on the normal development of the mice in the post-weaning period. These results substantiate previous findings on these animals, obtained by screening them in the pre-weaning 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.

Introduction

Most transgenic animals are produced by microinjection or embryonic stem (ES) 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 (Van Zutphen and Van der Meer, 1997; Mepham et al., 1998; Van der Meer and Van Zutphen, 1997). 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 (Rijkers et al., 1994).

We have started a study to identify and quantify physiological and behavioural differences in four groups of animals of the same mouse inbred strain differing only in their transgenic background (functional gene construct integrated, non-functional 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 behaviour (Desportes, 1985). It has become a reference strain for comparisons in various research fields such as, e.g., haematology 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 (Rogers et al., 1999).

The first part of the study emphasised the search for differences in the early postnatal development of the four experimental groups. To this end, newborn mice were subjected to various behavioural 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 (Van der Meer et al., 1999). The results of the pre-weaning study have indicated that the presence of the microinjected DNA construct (both functional and non-functional) 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 non-transgenic pups after transgenic procedures, compared to the control group. No significant differences in behaviour and/or morphological development were observed between the four groups in the pre-weaning period (Van der Meer et al., 1999).

In the present study, the same groups of mice are monitored in their post-weaning period, in order to compare different aspects of behaviour 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 post-mortem examinations were performed.

Animals and Methods

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 post-weaning development (age 4-30 weeks) and differed only in their transgenic backgrounds.

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 wildtype 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 fertilised 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 (PCR) with transgene-specific primers (for a detailed description of transgenic procedures, see Polites and Pinkert, 1994).

Group 2: non-functional 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 non-functional CRF construct. In the non-functional construct, no promotor 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 fertilised eggs (C57BL/6N Crl) and the same procedure was performed as described for Group 1; each subgroup was derived from a different founder male (non-functional CRF construct), which was crossed with a wildtype C57BL/6N Crl female.

Group 3: non-transgenic 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).

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 (Van der Meer et al., 1999). Each litter is from a different mother. For Groups 1a, 1b, 2a and 2b, only the transgenic animals were tested.

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 (Pine-wood ¾; 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 hours light: 12 hours dark cycle with lights on from 0600 hours to 1800 hours, 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*.

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.

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

Hole board test (exploration and habituation)
Exploratory behaviour was studied in a 16–hole board task (Boissier and Simon, 1962). The apparatus used has been described by Van de Weerd et al. (1994). 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 faeces and urine production was also registered for each mouse.

Cage emergence test (escape from novel environment)

In the cage emergence test (Van de Weerd et al., 1994), 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 faeces- and urine production were also recorded.

Behavioural profile as registered by LABORAS™

A newly developed behaviour registration system LABORAS™ (Laboratory Animal Behaviour Observation, Registration and Analysis System; Metris, Hoofddorp, The Netherlands) for the automated registration of different behavioural elements (Bulthuis et al., 1997; Schlingmann et al., 1998) was used for the following studies: (*a) 24-h behaviour observation, (b) 12-h extra climbing behaviour*, and *(c) 10-min light-dark test.* With a specially designed sensing platform, the position and the six behavioural 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 (Van de Weerd et al., 2001). 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 behavioural categories and automatically registered by a computer. Signals not recognised 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.

a) The 24-h behaviour observation

During 24 h, the behaviour 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 behavioural category) was calculated and analysed.

b) The 12-h extra climbing behaviour

Directly after the 24-h test, a metal climbing grid (size 16x10 cm, mesh size 0.5x0.5 cm) was vertically attached to the cage lid, to study differences in climbing behaviour after enrichment for the following 12 h (during the dark period). The relative mean time spent on climbing behaviour 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 behaviour test for the same animals.

c) Light-dark test (index of anxiety)
Anxiety-related behaviour was investigated in a light-dark test (Lister, 1990) using a cage specially adapted for LABORAS (Macrolon Type III cage, 38x22x27 cm, two equally sized compartments, one illuminated by 1000 lx). A clear Perspex tunnel (10x6x5 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.

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 standardised 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 behavioural response during this procedure. The scores used ranged from one to seven, as described by Van de Weerd et al. (1997). Several other responses of the animals (biting, freezing, or urine/faeces production) were also scored during the manipulation. Subsequently, the behaviour of the animals was observed in their home cage for 10 min using an instantaneous sampling method, meaning that every 5 s behaviour of the animal was noted according to a predefined ethogram, based upon Blom et al. (1992). The following behaviours are distinguished: immobility, locomotion, rearing, grooming, digging, climbing, eating, social behaviour, and fighting.

Post-mortem examinations
At the end of this study, the animals were killed and post-mortem 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.

Statistics
All data were statistically analysed 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 analysed by repeated-measurements analysis of variance (between and within ANOVA). For the behavioural measurements, repeated measurements ANOVA was also used for the hole board test, the (LABORAS) 24-h behaviour 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 analysed by one-way ANOVA. If ANOVA showed significant effects with respect to the behavioural 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. Behaviour performed by mice during the hole board test, the cage emergence test, and the handling test was analysed using non-parametric 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 pre-set at $P<0.05$ 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.

Results

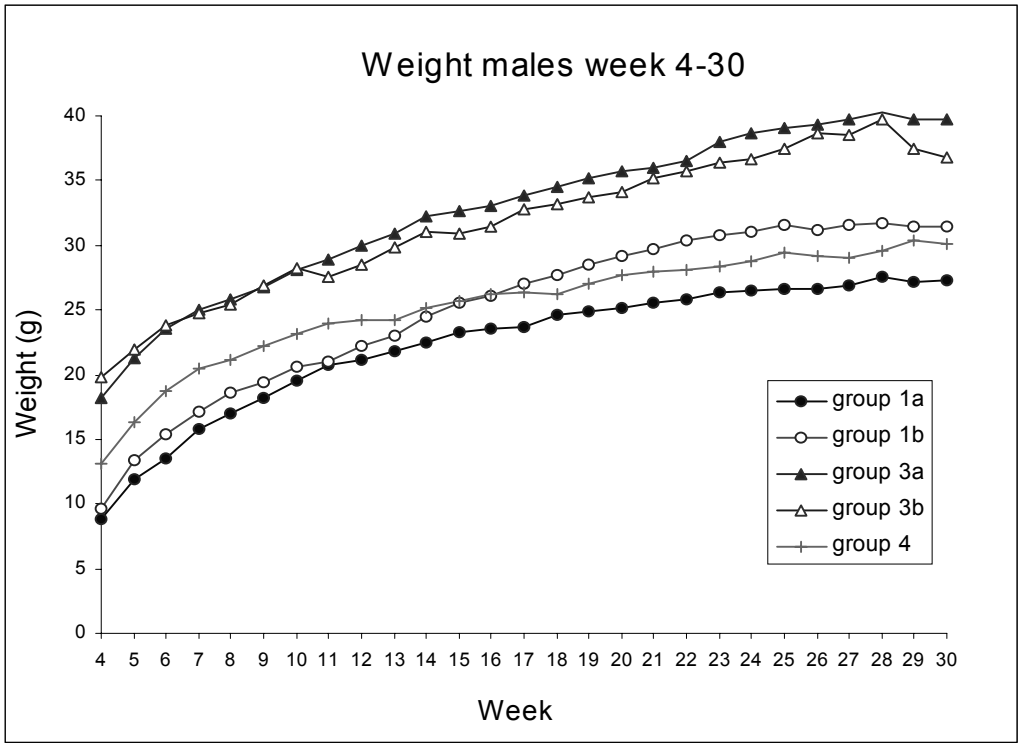
Survival rate

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 post-mortem examination of these mice.

Body weight/growth rate

Significant differences were found between groups ($F(6,120)=43.26$, $P<0.001$) and gender ($F(1,120)=142.34$, $P<0.001$) for body weight. Overall, males weighed significantly more than females ($P<0.001$) 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 ($P<0.001$). However, the weight gain of the mice of Groups 1a and 1b was significantly faster than the control Group 4 ($P<0.001$), 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 Figure 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 Figure 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 ($P<0.01$).



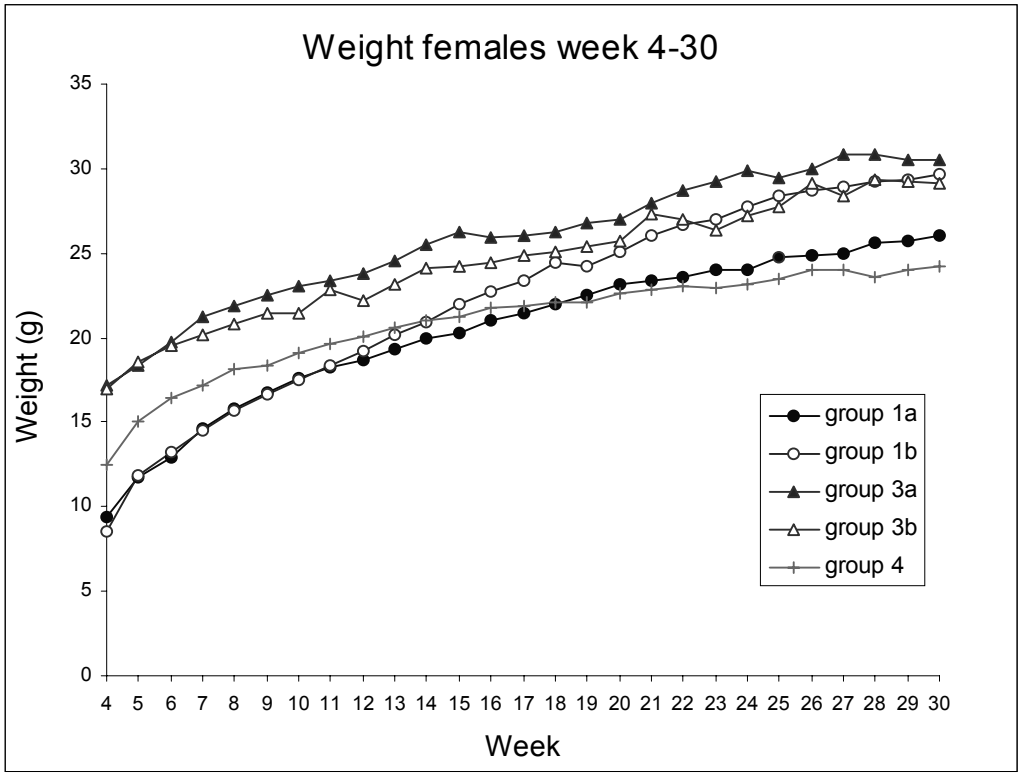


Figure 1: *Mean male (a) and female (b) body weight (g) in post-weaning period (age 4-30 weeks). No significant differences were found in mean body weight or growth rate between mice of Groups 2a and 2b compared to the control group (data not shown, omitted for clarity).*

Morphology/clinical appearance

All the CRF transgenic mice (Groups 1a and 1b) showed differences in morphology and clinical appearance: 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 pre-weaning period (Van der Meer et al., 1999). No differences in morphology or clinical appearance were detected for the other groups.

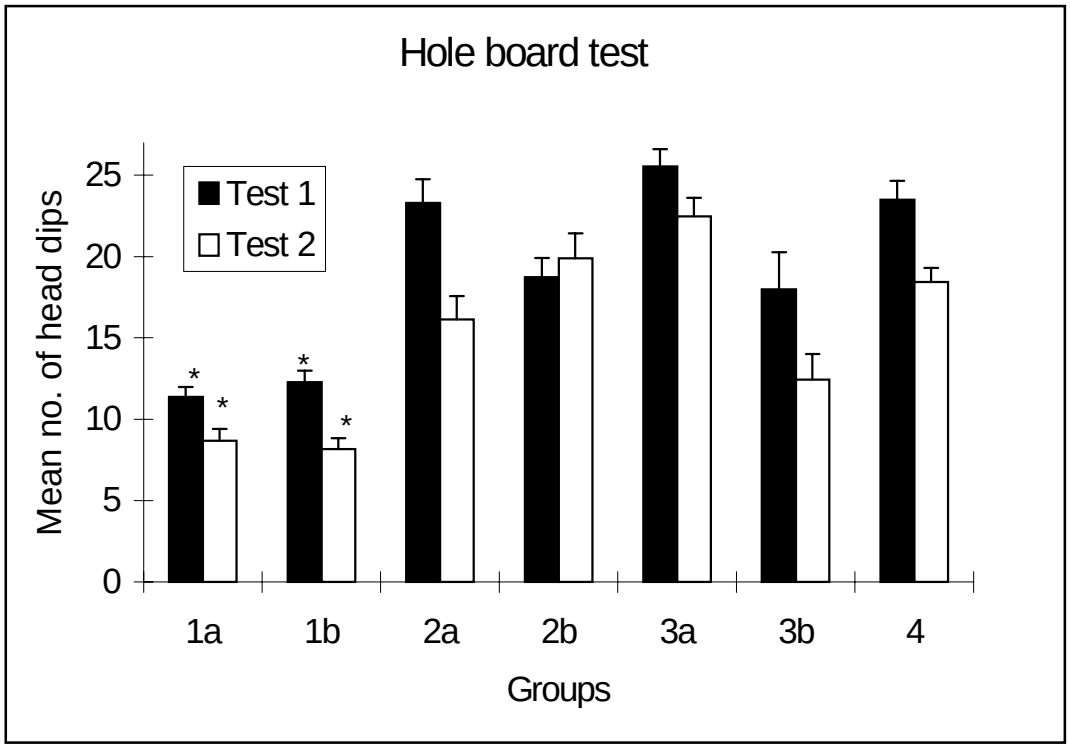


Figure 2: *Exploratory behaviour of the four test groups in two subsequent hole board tests. Group 1: CRF transgenics with functional CRF construct (1a and 1b represent progeny from two different founders), Group 2: CRF transgenics with non-functional CRF construct (2a and 2b represent progeny from two different founders), Group 3: non-transgenics 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 ($P<0.05$) based on ANOVA (repeated measurements) with main between-subject factors groups and sex and main within-subject factors Tests 1 and Test 2. Dunnett's post hoc tests with Bonferroni correction were used to study differences within groups. *$P<0.001$, significant difference compared to control group 4.*

Exploratory Behaviour

A significant effect of group ($F(6,123)=43.84$, $P<0.001$) on the number of holes explored in 3 min was detected. Figure 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 ($P<0.001$) or any other group ($P<0.01$). For all groups, effects for males and females were

similar, although overall females explored more holes than males ($F(1,123)=5.23$, $P<0.05$). There was a significant difference in number of holes explored in both tests between Groups 3a and 3b (Test 1: $P<0.01$; Test 2: $P<0.001$). All groups, except Group 2b, showed a significant decrease in number of holes explored in Test 2 compared to Test 1 ($F(1,123)=52.57$, $P<0.001$). 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 ($P<0.002$). During the first hole board test, the faeces production of the Groups 1a, 1b, and 2b was significantly higher than the control group ($P<0.01$).

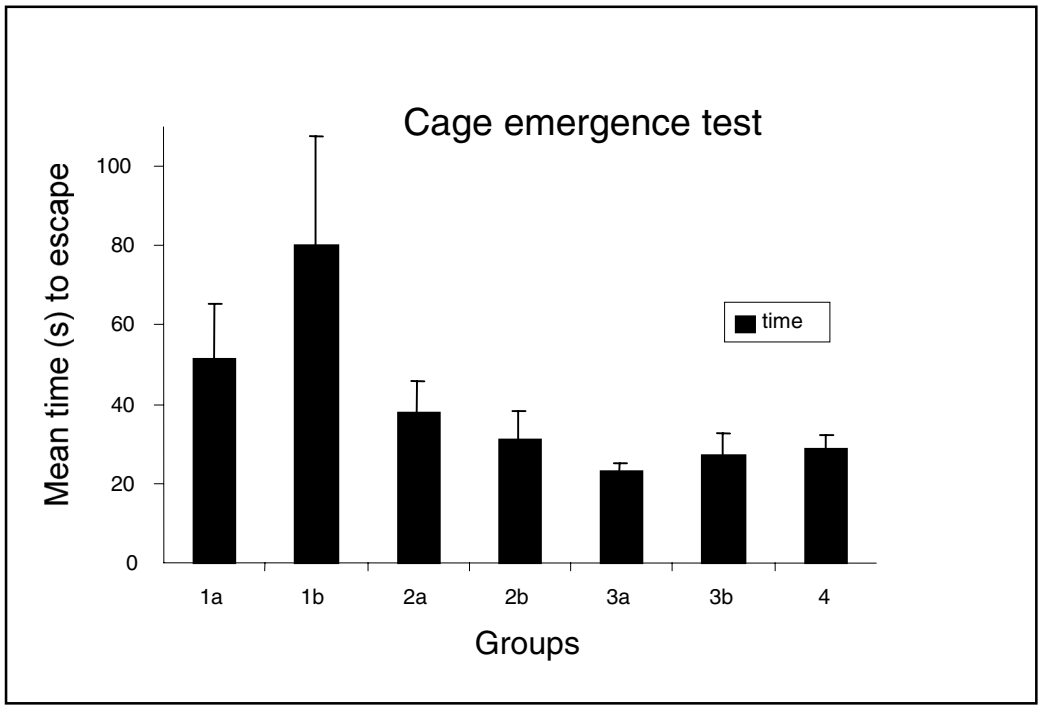


Figure 3: *Mean time (sec ± S.E.M.) to escape from an empty cage.*
Testing was performed at the age of 16 weeks between 1500 and 1700 h. Maximum testing period was 10 min. Differences between treatment groups and control group analysed by one-way ANOVA (ns).

Cage emergence test

For the cage emergence test (Figure 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 behaviours scored during the test. The behaviours most frequently observed were rearing to the sidewalls and sniffing at the hole.

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 whether the test started by placing the animals in the dark or

in the light compartment ($F_{latency}(6,123)=15.34$, $P<0.001$; $F_{light}(6,117)=2.18$, $P<0.01$; $F_{dark}(6,117)=3.69$, $P<0.01$; $F_{crossings}(6,122)=19.99$, $P<0.001$; respectively, see Figure 4). Gender effects were only observed for the number of crossings ($F(1,122)=10.13$, $P<0.01$).

Latency until the first entry in the other compartment was increased for Groups 1a and 1b when compared to controls ($P<0.001$) and to the other groups in both tests (1a vs. 2a, 2b, 3a, 3b, and 4: $P<0.01$; 1b vs. 3a, 3b, and 4: $P<0.001$). Overall, when started from the light compartment, latency is higher (Figure 4a) than when started from the dark compartment (Figure 4b). This difference is mainly caused by the transgenic mice of Groups 1a and 1b ($P<0.01$, 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 (Figure 4b). Total time spent in the light is only significant shorter for Groups 1a and 1b compared to the control group ($P<0.01$) and the other groups ($P<0.05$).

When starting from the light compartment (Figure 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 the light compartment than the control group, $P<0.01$).

Overall, the number of crossings (Figure 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 ($P<0.001$). 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 ($P<0.01$, 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, $P<0.05$).

LABORAS 24-h test

Figure 5 presents the results of the 24-h behaviour as recorded by LABORAS. Per time period, the mean percentage of time spent on each of the six different behaviour 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 behaviour. Significant differences in behavioural patterns were found between transgenic CRF animals (Group 1b) and the control group (mainly during the dark period).

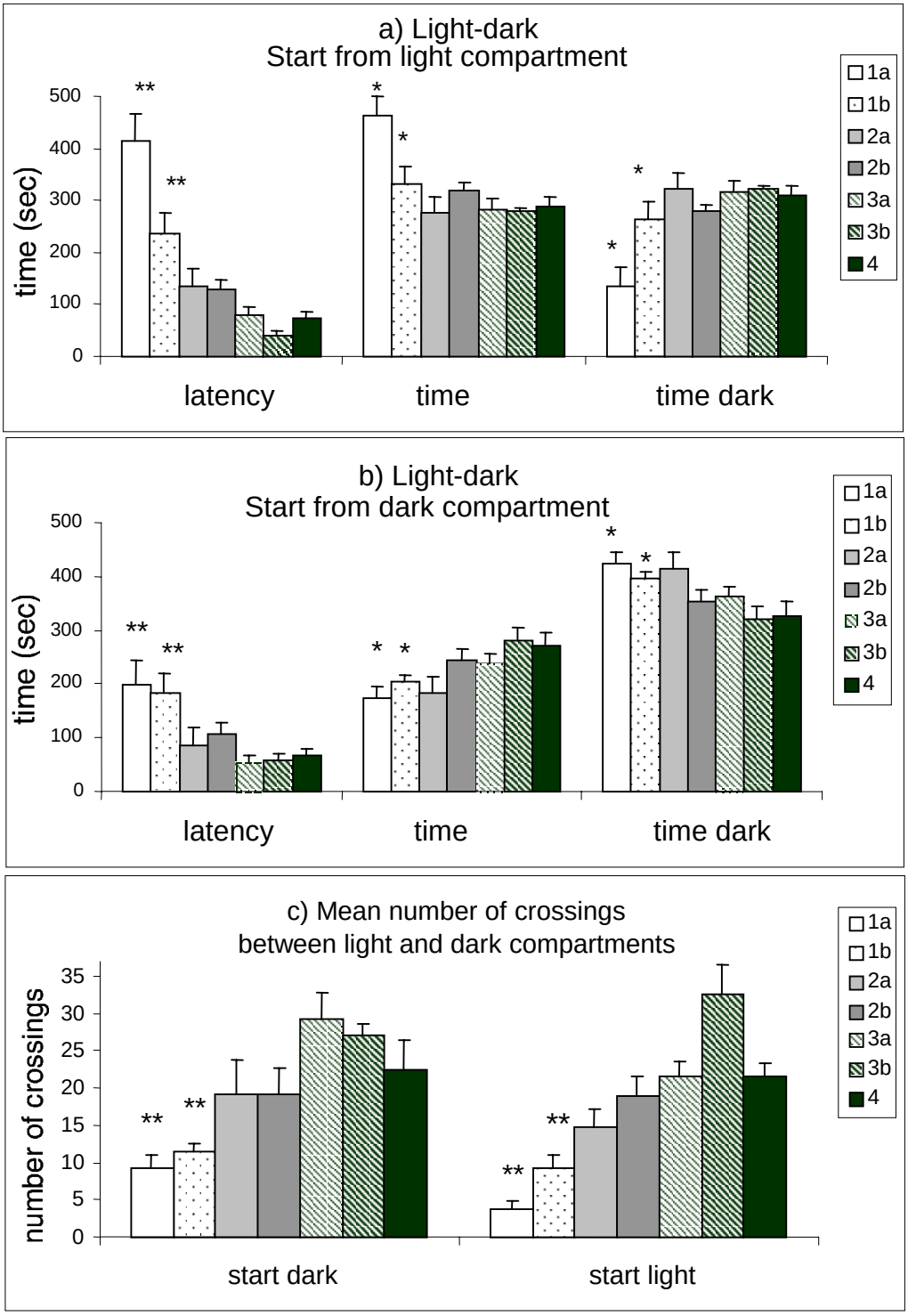


Figure 4: *Results of the light-dark preference test. (a) Light-dark test, start from light compartment, (b) light-dark test, start from dark compartment, (c) mean number of crossings between the two compartments.*
Half of the number of the animals of each group was placed in the light compartment (a) 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 ($P<0.05$) based on two-way ANOVA with main factors group, sex and input in dark or in light compartment.
**$P<0.01$; **$P<0.001$, significant difference compared to the control group 4.*

Immobility (Figure 5a)
Overall, all groups ($F(4,86)=6.74$, $P<0.001$) except Group 3b, spent more time on immobility than the control animals (1-24 h, Groups 1b and Group 3a: $P<0.005$, Group 2b: $P<0.05$). For the first two periods (1-3 h and 4-6 h), only the CRF transgenic animals showed higher percentages of immobility ($P<0.05$ and $P<0.01$), while for the 7-9 h and 10-12 h periods, the immobility of the other groups was also significantly higher compared to the controls ($P<0.005$). During the light period, no significant differences in duration of immobility were found between the groups.

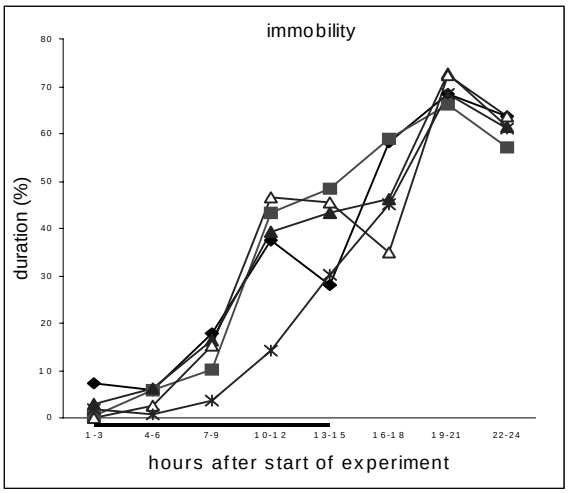
Locomotion (Figure 5b)
The transgenic CRF animals showed significant less locomotion compared to control group, mainly during the dark period (ANOVA group effect: $F(4,86)=10.25$, $P<0.001$; Group 1b vs. controls: $P<0.01$).

Climbing (Figure 5c)
Overall, the CRF transgenic mice spent significant less time climbing compared to the control group (ANOVA group effect: $F(4,86)=25.04$, $P<0.001$; 1-24 h, Group 1b vs. controls: $P<0.005$). In the first four periods (dark period), the mice of Group 3a also showed less climbing behaviour ($P<0.05$) 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 ($F(1,86)=49.70$, $P<0.001$).

Grooming (Figure 5d)
Overall, ANOVA revealed a significant group effect for grooming ($F(4,86)=4.08$, $P<0.01$). For the 1-3 h, 4-6 h and 7-9 h periods, animals of Group 1b ($P<0.05$) and Group 3a ($P<0.01$) showed significant more grooming compared to the controls, while for the 10-12 h period, this was only significant for Group 3a ($P<0.01$). For the other periods, no significant difference was found.

Drinking (Figure 5e)
For the 1-3 h ($P<0.005$), 4-6 h ($P<0.005$) and 7-9 h ($P<0.01$) periods, the CRF transgenic mice showed significant more drinking behaviour compared to the control group (ANOVA group effect: $F(4,86)=3.01$, $P<0.05$).

Eating (Figure 5f)
All groups of mice showed a significantly reduced eating behaviour when compared to the control group (ANOVA group effect: $F(4,86)=15.40$, $P<0.001$; Group 1-3 vs. controls: $P<0.005$). This was most evident during the dark period ($P<0.05$).



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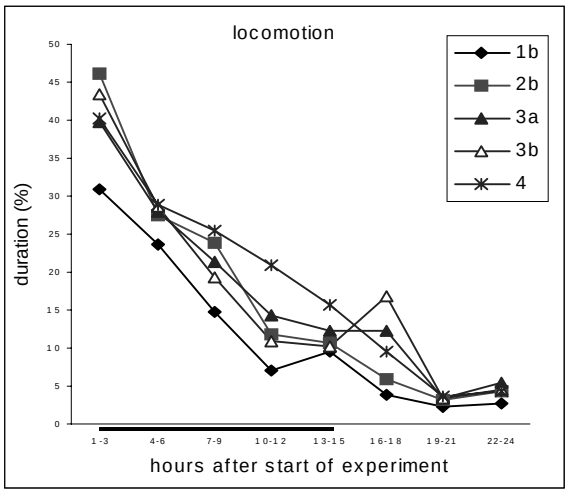

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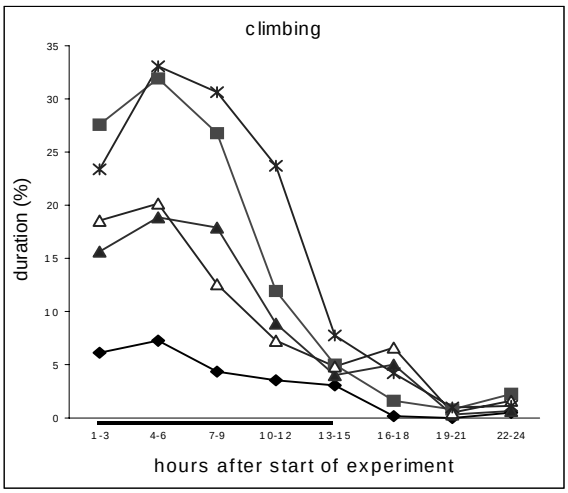

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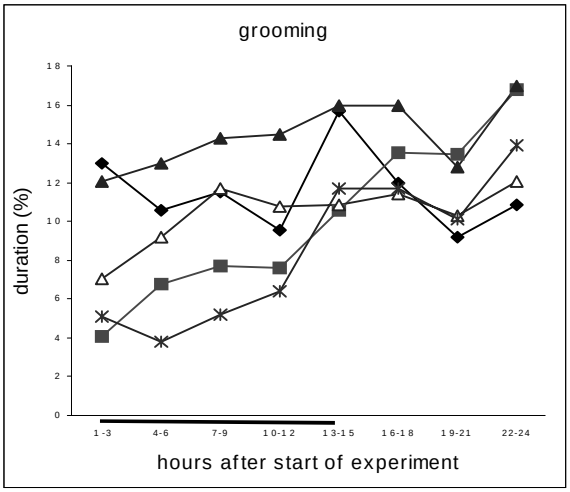

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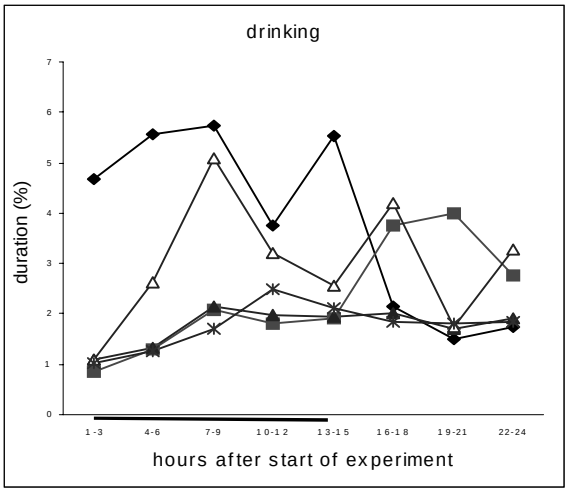

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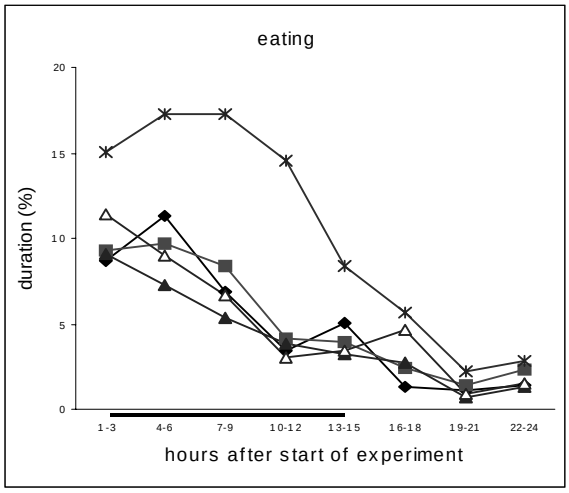

5f

Figure 5: *Results of the LABORAS behaviour registration system, 24-h test.*
Per time period of 3 h, the relative mean time spent on each of the six behavioural 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 ($P<0.05$) 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.

LABORAS 12-h extra climbing

Directly after the 24-h test, the animals were tested for extra climbing behaviour by adding an extra climbing grid to the cage. During the following 12 h (dark period), the climbing behaviour was recorded. Overall, females spent more time on climbing than males for all groups ($F(1,86)=25.54$, $P<0.001$). This difference is less significant for the transgenic mice of Group 1b, where both males and females showed very little climbing behaviour (data not shown). Climbing behaviour 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 behaviour for the 4-6 h and the 10-12 h periods. Overall, mice of all groups did not spend significantly more time on climbing when the extra climbing grid was added to their cage.

Handling test

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

Post-mortem examinations

Significant differences were found for the adult body weight between groups ($F(6,65)=14.59$, $P<0.001$) and gender ($F(1,65)=25.99$, $P<0.001$) at the post-mortem examination (Table 1, see also Figure 1). The males of Groups 3a and 3b were heavier compared to the controls ($P<0.001$) and the males of Group 1a were lighter ($P<0.05$), while the females of Groups 1b, 2a, 3a and 3b were all heavier than the controls ($P<0.05$). 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: $F(6,65)=8.02$, $P<0.001$; Groups 1a and 1b vs. controls: $P<0.01$). After analysis of the relative spleen weight, this decrease was also significant for males of all other groups (ANOVA group effect $F(6,32)=3.41$, $P<0.05$; Group 1-3 vs. controls: $P<0.05$), 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: $F(6,32)=5.46$, $P<0.01$; Group 3 vs. controls: $P<0.05$). 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 Group 3 (ANOVA group effect: $F(6,65)=8.94$, $P<0.001$, Groups 2 and 3 vs. controls: $P<0.05$) and absolute liver weights were increased for Groups 2a and 3 compared to the controls (ANOVA group effect: $F(6,65)=5.93$, $P<0.001$: Groups 2a and 3 vs. controls: $P<0.05$).

***Table 1**:*

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

Organ	Group 1a	Group 1b	Group 2a	Group 2b	Group 3a	Group 3b	Group 4
Body weight							
Males (g)	24.99±1.62 [c]	30.41±1.76	38.02±2.84	35.79±1.84	43.70±1.76 [a]	42.45±1.18 [a]	31.77±0.93
Females (g)	23.31±0.54	33.02±1.76 [c]	34.08±3.65 [c]	27.54±1.76	33.65±1.25 [c]	33.06±0.79 [c]	24.63±0.57
Spleen							
Males	5.00±0.45 [b]	5.50±0.22 [b]	7.60±0.60	8.50±0.50	7.33±0.56	7.50±0.65	10.83±2.51
Females	5.00±0.52 [b]	6.17±0.40 [b]	8.71±0.68	13.67±2.20	10.17±0.95	9.00±0.69	9.17±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 [c]	25.17±1.30 [c]	25.17±0.60 [c]	24.75±0.63 [c]	22.33±1.09
Females	17.67±0.56	18.83±0.79	19.86±1.39 [c]	21.33±0.99 [c]	22.67±1.20 [c]	21.33±1.20 [c]	16.67±0.80
Liver							
Males	115±11.13	149±11.31	194±16.8 [c]	185±13.50	226±34.35 [c]	218±15.72 [c]	158±12.97
Females	125±4.29	151±6.44	165±13.22 [c]	126±10.82	147±10.96 [c]	159±5.78 [c]	120±5.74

Values are means of six males or six females per group (± S.E.M., in grams x10^{-2}), except for Group 2a (five males and seven females) and Group 3b (four males and three females). Body weights are shown in grams. Significance (P<0.05) based on two-way ANOVA with main factors group, sex, and absolute or relative organ weight. [a]P<0.001, [b]P<0.01, and [c]P<0.05, significant difference compared to the control group.

Post-mortem 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 tumour 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.

Discussion

This study evaluated the impact of biotechnological procedures involved in the production of transgenic animals on the welfare of these animals during their post-weaning development by measuring various behavioural, 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 post-weaning 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 post-weaning period than the controls, but they did not show a higher growth rate. The transgenic animals with a non-functional construct (Groups 2a and 2b) did not differ significantly in their body weight from the controls during both the pre- and post-weaning 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 wildtype mice). Several of these features are obviously due to increased corticosterone levels, caused by the overexpression of the CRF gene (Stenzel-Poore et al., 1994; Boehme et al., 1997).

The hole board test represents a novel environment of increased structural complexity (Takeda et al., 1998). It is designed to test exploratory behaviour, as it takes advantage of the natural tendency of mice to dip their heads into holes (Lister, 1990; File and Wardill, 1975). In both hole board tests, the transgenic CRF mice were hypoactive compared to all other groups. During the first test, they also produced more faeces 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. (1971), 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 behaviour 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 (Crawley, 1981; Crawley and Davis, 1982). 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 (File, 1992). 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 behaviour observations showed similar behavioural circadian patterns for Groups 2-4. High levels of activity associated with exploration (Saibaba et al., 1995) 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 behavioural patterns are consistent with circadian rhythms of mice as found by others (Weinert, 1994; Schlingmann et al., 1998; Van de Weerd et al., 2001). 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 (Lubberink, 1977; Meij, 1997).

The control mice showed more eating behaviour compared to the other groups, although their body weight did not increase. Eating behaviour 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 post-mortem examinations. The absolute weight of the spleen was decreased for the transgenic CRF mice compared to the control group. Boehme et al. (1997) also found that after adrenalectomy, the size and weight of the spleen of transgenic CRF mice recovered to nearly normal.

Conclusion

The tests described in this chapter were primarily designed to estimate the effects of biotechnological procedures of transgenesis on the development of the animals in the post-weaning period (Weeks 4-30) of mice. In the present study, differences in behaviour, body weight, and morphology between animals with CRF transgenic expression (Group 1) and the control group were found. Behaviour of transgenic CRF mice differed significantly from control animals in test situations designed to assess behavioural activation and anxiogenic-like states. They showed less exploratory behaviour 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 behaviour. 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 (pre-weaning period) substantiate these observations (Van der Meer et al., 1999). 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 relative 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 behavioural 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 behavioural phenotyping of other newly produced transgenic lines and to study possible effects on their welfare.

Acknowledgements

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Chapter 4

Consequences of gene targeting procedures for behavioural responses and morphological development of newborn mice

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Summary

In this study the effects of gene targeting procedures on the early behaviour and morphological development of the resulting offspring have been investigated. Six groups of mice, each having undergone a specific aspect of the biotechnological procedure, (including electroporation, microinjection and/or embryo culture) and one control group, were compared. Development of behaviour, morphological characteristics and body weight of the progeny were tested daily from birth to weaning (0-3 weeks) for all groups. No significant differences in behaviour or morphological development were observed. However, the occurrence of increased (perinatal) pup mortality and increased body weight in the procedural groups, indicates that during the production of gene targeted mice, some of the normal physiological and/or developmental processes can be affected. Therefore, gene targeting procedures should always be accompanied by careful monitoring of health and welfare of the resulting offspring.

Introduction

Several methods are used to produce transgenic animals. The most frequently used ones are either microinjection, where the gene construct is injected into one of the pro-nuclei of the fertilised egg (Gordon et al., 1980; Gordon and Ruddle, 1981; Moore and Mepham, 1995) or embryonic stem (ES) cell-mediated gene targeting, where the transgene is precisely targeted by homologous recombination (Capecchi, 1989a; Robertson, 1991; Wilder and Rizzino, 1993). In previous studies, the effects of the microinjection procedures on behaviour and physiological/morphological development of the resulting offspring were studied (Van der Meer et al., 1999, 2001a). In the present study, the effects of the ES cell-mediated gene targeting procedures are investigated. Briefly, ES cells are derived from the inner cell mass of murine blastocysts and remain undifferentiated under suitable tissue culture conditions. By using DNA constructs designed to homologously recombine (exchange genetic information) with regions of identical sequence on a chromosome in the ES cells, the ES-cell technique enables the site-directed insertion/exchange (knock-in), or mutation/deletion of specific endogenous genes (knock-out). The most common way to introduce the transgene into the ES cells is by electroporation, using short, high voltage electrical pulses. These pulses cause nanometer-sized pores in the plasma membrane, allowing the penetration of the transgenes into the cytoplasm of the ES cells (Chu et al., 1987). Selection procedures are then used to isolate ES cells that have incorporated the transgene into their genome and stable cell lines can be established from single, genetically modified ES cells. These ES cells are then microinjected into blastocysts of a donor animal and these are transferred to foster mice to develop to term. Several of these blastocysts develop into genetically chimeric animals, being composed of cells derived from the host blastocyst and from the injected ES cells (usually visualised by mixed coat colours). Non-chimeric mice will be genetically normal (that is, do not contain the transgene in any of their cells), due to the failure of the ES cell component to fully participate in the process of foetal development. A number of the chimeric mice may be germ-line chimeras, with the ability to produce gametes carrying the ES-cell derived transgene; they can be used for further breeding to produce a homozygous transgenic line, in contrast to the non germ-line chimeras.

During gene targeting, cells and embryos are subjected to various *in vitro* manipulations, including electroporation, microinjection, *in vitro* embryo culture and embryo transfer. Until now, the effects of these manipulations have not been studied. In particular, the consequences for health and welfare of the resulting offspring, either during gestation, or as young or adult animals, 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 I), in order to determine whether the manipulations had any effect on the development or behaviour of the progeny. Specific effects of electroporation, microinjection and embryo culture on the development of (chimeric) mice could be studied this way. The study was performed in parallel to a project in which the possible role of APC2, a homologue of the adenomatous polyposis coli (APC) tumour suppressor protein, in development is being studied (Van Es et al., 1999). The APC2 knockout construct has been introduced by

electroporation into ES cells, and both the chimeric and non-chimeric mice were used for our study.

This study focuses on the search for differences in the early postnatal development of the experimental groups compared with control animals (C57BL/6). To this end, newborn mice were subjected to various behavioural tests and their growth and development of morphological characteristics were recorded daily from birth up to the age of 3 weeks. A test protocol was developed for the systematic observation of the animals and for collecting information, which might be relevant for establishing the impact of gene targeting procedures on the welfare of these animals (Van der Meer et al., 1999).

Animals and Methods

Animals

Six groups of mice, all with a different gene targeting background and one control group, were used for this study (see Table I). The control mice and all the blastocysts used were from the same (black) inbred strain (C57BL/6 JIcoU; Central Laboratory Animal Institute, CLAI, Utrecht University, Utrecht, The Netherlands). The embryonic stem (ES) cells used (E14, ref. Hooper et al., 1997; provided by A. Berns of the Netherlands Cancer Institute, Amsterdam) were derived from the 129/Ola (agouti) strain. All foster mothers were B6D2F_1/CrlBR (Charles River, Sulzfeld, Germany). The gene targeting techniques were performed by an experienced technician according to standard procedures (Verbeek et al., 1995; Schilham et al., 1996; Hazenbos et al., 1996). All animals were tested during the same period of pre-weaning development (age 0-21 days).

C mice: control animals, no gene targeting procedures

These animals were normal C57BL/6 JIcoU mice, not submitted to any gene targeting procedure. A total of 22 pups (13 males and 9 females; 5 litters) were tested.

B mice: untreated blastocysts transferred to foster mothers

Blastocysts were isolated, cultured and transferred to foster mothers without any further gene targeting treatment. A total of 18 progeny mice were available for testing (10 males and 8 females; 5 litters). B mice were used to study the effects of blastocyst manipulation.

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. A total of 19 progeny mice (12 males and 7 females; 5 litters) were available for testing. BSh mice were used to study the effect of the microinjection procedure on blastocysts.

Ch+ mice: chimeric animals after electroporation of ES cells with APC2 knockout gene
The APC2 mouse gene construct (kindly provided by Van Es et al., 1999) was introduced into the 129/Ola derived ES cell line by electroporation (250 V, 500 µ, 7.8 ms) using standard procedures (Verbeek et al., 1995). After marker-based selection, positive targeted clones were used for microinjection into the blastocysts. Chimeric mice (as identified by coat colour) were obtained by injecting 10-15 gene-targeted ES cells into C57BL/6 blastocysts. An average of 10-12 injected blastocysts were transferred into pseudo-pregnant females (recipient mice). A total of 14 chimeric pups were tested (7 females, 7 males; 6 litters). Ch+ mice were used to study effects of the gene targeting procedure in chimeric mice that are carrying the mutant gene.

Ch- mice: non-chimeric animals after electroporation of ES cells with APC2 knockout gene
Non-chimeric animals after procedures performed for the production of the Ch+ chimeras, as identified by their black coat colour. A total of 9 pups were tested (6 males and 3 females; 6 litters). In Ch- mice the ES cells have not contributed to the phenotype. These animals are comparable to BSh mice.

NCh+ 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 coat colour) were obtained by injecting 10-15 ES cells into C57BL/6 blastocysts. An average of 10-12 injected blastocysts were transferred into pseudo-pregnant females (recipient mice). A total of 14 pups were tested (8 males, 6 females; 7 litters). NCh+ mice were used to study the effects of chimerism (without the effect of the mutated gene).

NCh- mice: non-chimeric animals without electroporation of ES cells
Non-chimeric animals after procedures performed for the production of the NCh+ chimerics (as identified by their black coat colour). A total of 15 pups were available for testing (9 females, 6 males; 7 litters). NCh- served as controls for the chimeric NCh+ mice.

***Table I**: Biotechnological procedures*[a]

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

[a]*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 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 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-=non-chimeric).*

Housing and handling conditions

The pregnant C57BL/6 JIcoU mice were individually housed in wire-topped Macrolon Type II cages (410 cm^2, Tecniplast Inc., Rome, Italy) with sawdust bedding (Pine-wood 3/4; Woodyclean, BMI, Helmond, The Netherlands). Per cage a tissue (Kleenex, Kimberley-Clark) was added for nest building. The ($B6D2F_1$/CrlBR) foster mothers were individually housed after the implantation procedure. Animals were housed conventionally and maintained under standard conditions (12:12h light: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*.

Details of pup handling have been described previously (Van der Meer et al., 1999). In short: on the day of birth (day 0), the pups were weighed and inspected for any malformations or special traits. For identification purposes, pups were marked with a non-toxic blue dye (Pentel felt pen F50, permanent marker). For each litter, the number of pups found dead was recorded daily from day 0 until day 21. During the first 5 postnatal days, pups were inspected for the presence of a milk spot (a white spot in the stomach) as an indicator for milk uptake. Mice were weaned at the age of 3-4 weeks. Pups were weighed daily (day 0-21) and subjected to a set of behavioural tests and morphological screenings. Testing took place in a sequence that varied randomly over groups.

Behavioural tests

A modified version of the test battery developed by Fox (1965) and Altman and Sudarshan (1975) was used in order to screen for possible behavioural abnormalities in each of the categories of animals (Costa, 1997a). For details see van der Meer et al. (1999). In short, four levels of response to these tests could be distinguished: 0 (if behaviour or response is absent), 1 (signs of primitive response), 2 (a clear but not yet mature response) or 3 (a mature and full response in all aspects of execution such as co-ordination or strength).
The animals have been submitted to the following tests.

Surface righting (labyrinth and postural response)

The test was performed by placing the pups on their back with the limbs pointing upwards. The maximum response was to turn over immediately and stand on all four limbs.

- Level 0: no response;
- Level 1: the animal lies on its back, moving its paws in the air without any direction, and there is no righting;
- Level 2: the animal rights itself, but slowly; the direction of the paws is more controlled in the righting direction;
- Level 3: the animal rights itself immediately and stands on its four paws.

Walking (co-ordination and muscular strength)

This includes the testing of the development of locomotion.

- Level 0: no locomotion;

- Level 1: pivoting - moving around with the help of the head and forelimbs, but not using the hind limbs;
- Level 2: crawling - moving on all four limbs, dragging the belly over the surface;
- Level 3: walking - mature locomotion with the body supported completely by the four limbs.

Cliff drop aversion test (somato sensory response)

The pups were individually placed on the edge of a surface of polystyrene foam (height 3cm) with the forelimbs and front of the head partially off the foam. The mature response is that the animal quickly turns its head and forelimbs to avoid dropping.

- Level 0: animal falls off the foam;
- Level 1: the animal turns very slowly back to the surface, without falling off the foam;
- Level 2: the animal avoids the cliff, but it still takes some time to turn;
- Level 3: the animal immediately turns back.

Negative geotaxis test (labyrinth and postural reaction)

The pups were placed in a head-down position on a surface of polystyrene foam which was inclined at a slope of 45°. The mature response is when the animal immediately rotates 180° to the head-up position and starts to climb up the slope.

- Level 0: the animal does not move at all;
- Level 1: the animal turns its body slowly up the slope, but stops halfway without moving any further;
- Level 2: the animal turns its body, stops halfway and then continues turning up to 180°;
- Level 3: the animal turns immediately by 180° and moves towards the top of the slope.

Grasping test (freeing reflex)

Occurs when the inside of the forepaw is stimulated with a thin cotton bud. The mature response is when the animal immediately grasps it. This reflex disappears with the development of the nervous system.

- Level 0: no grasping;
- Level 1: the animal puts its paw on the cotton bud, but it does not hold on firmly;
- Level 2: the animal puts its paw on the cotton bud with more force, but when the cotton bud is pulled, cannot hold it;
- Level 3: the animal grasps the cotton bud very firmly.

Bar holding test (muscular strength)

The pup has to grasp a bar (cotton bud) and its ability to hang on to the bar is observed. The mature response is when the animal holds the bar with all four limbs, with its tail wound around the bar.

- Level 0: animal cannot hang on to the bar;
- Level 1: animal holds on to the bar with forepaws only;

- Level 2: animal can put its hind paws on to the bar, but not its tail;
- Level 3: animal puts all its four paws and tail around the bar.

Morphological screening

Morphological development, such as the age of onset of incisor eruption, nipple development, eye-opening, ear-elevation and opening, and hair growth was determined by daily (days 0-21) observation of the individual animals.

Hair growth

- Level 0: no hair present, animals are still bald;
- Level 1: fur starts as fine stubble over the back;
- Level 2: complete coat of fine fuzzy fur is visible;
- Level 3: animals are totally covered with thick hair.

Incisor eruption (lower and upper incisor)

- Level 1: incisors are visible, but not erupted;
- Level 3: incisors are erupted.

Eye opening

- Level 1: eyelids start to open, slit-like palpebral opening;
- Level 3: eyelids are totally open, oval palpebral opening.

Ear elevation

The ears of newborn pups lie flat against the head. A score of 3 is given when both ears are elevated 45° away from the head.

Ear opening

- Level 1: ears start to open, slit-like opening;
- Level 3: ears are totally open.

Statistical analysis

All data from the various observations were statistically analysed (by using SPSS 9.0 as statistical package), to determine any significant difference between the treatment groups and the control group. For the behavioural and morphological parameters, data were evaluated by using non-parametric statistics: Kruskal-Wallis test, and, where appropriate, followed by non-parametric multiple comparisons tests (Siegel and Castellan, 1988). For all the parameters the level of statistical significance was pre-set at $P \leq 0.05$. The body weight results of the pups during the pre-weaning period were analysed by repeated measurements analysis of variance (ANOVA). For the statistical analysis, data were used from all the pups that survived day 2. Statistical analyses were performed using the litter as the unit of measurement, by computing the mean value of data for pups in each litter.

Results

***Table II**: Survival rates of mouse litters in the various test groups[a] (day 0-21)*

	C	B	BSh	Ch+/Ch-	NCh+/NCh-
No. of (foster) mothers	5	5	6	16	11
No. of litters[b]	5	5	5	6	7
No. of offspring	23	20	22	27	32
Litter size at testing[c]	4.4±1.82	3.6±1.14	3.8±0.84	3.8±0.98	4.3±1.50
Pups dead[d]	1	2	3	4	3
Chimerics[e]	-	-	-	59.0%	51.7%

[a] *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+: Chimeric animals after electroporation with APC 2 knockout gene (B6/129)
Ch-: Non-chimeric animals after electroporation with APC 2 knockout gene (B6)
NCh+: Chimeric animals without electroporation (B6/129)
NCh-: Non-chimeric animals without electroporation (B6)

[b] *One foster mother of group BSh and six foster mothers of group Ch+/Ch- did not get pregnant after implantation of the blastocysts. Moreover, four foster mothers of both Ch+/Ch- and NCh+/NCh- groups produced only one pup which died on the day of birth (day 0).*
[c] *Mean ± SD.* [d] *Pups died on day 0, day 1 or day 2. Dead pups were not tested for chimerism. In both the Ch+/Ch- and NCh+/NCh- groups, four foster mothers had only one pup that died on day 0 (excluded from Table II).* [e] *Percentage based on pups alive after day 2.*

Litter data

The numbers of litters used in various developmental and behavioural analysis were respectively 5, 5, 5, 6, and 7 in control C and B, BSh, Ch+/Ch- and NCh+/NCh- groups (see Table II, chimeric and non-chimeric mice were in the same litters). There was no significant difference in the average litter sizes at testing (days 1-21) between the treatment groups and the control group (Table II). However, in the Ch+/Ch- group, 10 out of 16 foster mothers had either no litter (n=6) or only one pup that died at day 0 (n=4). In the NCh+/NCh- group, 4 out of 11 foster mothers had only one pup that died on day 0.

Overall, there was no significant effect of group for the survival rates. If the foster mothers with one pup that died at day 0 are taken into account, both Ch+/Ch- (8/31) and NCh+/NCh- (7/36) groups had a higher, marginally significant ($P \leq 0.06$) pup mortality compared to the control group C (1/23). For the other groups, no differences could be demonstrated in pup survival rates. In all groups, pup mortality occurred before postnatal day 3 and was distributed over separate litters.

The numbers of pups available for testing were 22, 18, 19, 14, 9, 14, and 15 for groups C, B, BSh, Ch+, Ch-, NCh+ and NCh-, respectively.

Weight curve

Figure 1 shows the mean pup weights during the experiment. Repeated measures ANOVA revealed significant group effects ($P<0.001$) for body weight, but no effects for gender were detected. Birth weights (day 0) were significantly higher for the chimeric Ch+ ($P<0.05$) and NCh+ mice ($P<0.01$), and also for the non-chimeric Ch- and NCh- mice ($P<0.01$), when compared to the controls C, B and BSh mice (see Table III). Analysis of the development of body weight over the rest of the pre-weaning period indicated a significantly higher average weight for all treatment groups when compared to the control group C. Multiple comparisons demonstrated no further significant differences in body weight between the other groups. All pups that died before postnatal day 3 had a body weight which was lower than their litter average body weight.

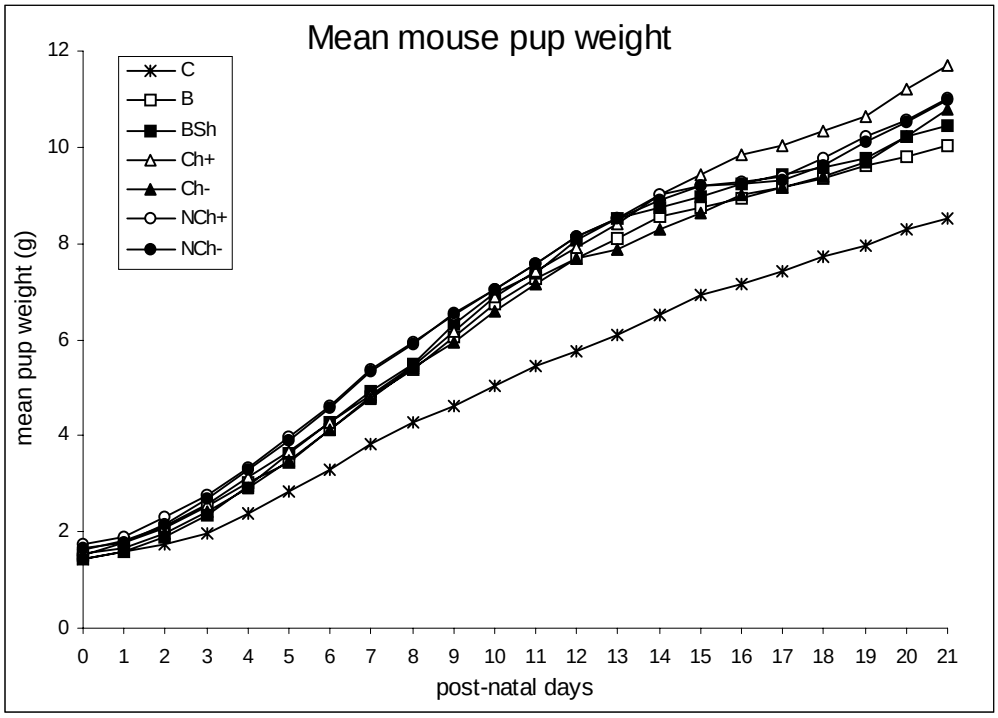

Figure 1: *Mean mouse pup weight in pre-weaning period.*
All pups from the various groups were weighed daily from day 0 until day 21.

Table III*: Birth weight data*

Group	C	B	BSh	Ch+	Ch-	NCh+	NCh-
Birth weight	1.45 ± 0.10	1.50 ± 0.08	1.45 ± 0.16	1.64 ± 0.14 [b]	1.66 ± 0.08 [a]	1.74 ± 0.13 [a]	1.67 ± 0.15 [a]

The birth weight data of the different groups are shown ([a]P<0.01; [b]P<0.05 significantly different compared to the controls C, B and BSh mice).

Behavioural observations

The results of the different behavioural variables are presented in Table IV. Overall, developmental retardations were not found for any group in any of the behavioural tests. All animals reached adult scores (level 3) for walking, cliff-drop aversion, negative geotaxis, righting, grasping, and bar holding during the test period (0-3 weeks) in all treatment groups.

***Table IV**: Summary of behavioural tests in mice*

Test	C	B	BSh	Ch+	Ch-	NCh+	NCh-
Walking							
Level 1	3.59 ± 0.67	3.28 ± 0.46	3.37 ± 0.50	3.86 ± 0.36	3.56 ± 0.53	3.40 ± 0.51	3.27 ± 0.59
Level 2	6.59 ± 0.59	6.61 ± 0.61	6.79 ± 0.54	6.36 ± 0.74	6.00 ± 0.00 [a]	6.93 ± 0.70	7.20 ± 0.41 [a]
Level 3	9.00 ± 0.69	9.39 ± 0.50	9.21 ± 0.54	8.71 ± 0.91	8.78 ± 0.83	9.33 ± 0.72	9.80 ± 0.56
Period	5.41 ± 0.73	6.11 ± 0.47	5.84 ± 0.83	4.86 ± 1.03 [b]	5.22 ± 0.83	5.93 ± 1.03	6.53 ± 0.64 [b]
Cliff drop							
Level 1	3.82 ± 0.39	3.50 ±0.51 [ab]	4.00 ± 0.33	4.14 ± 0.36	4.00 ± 0.50	4.67 ± 0.82 [a]	4.60 ± 0.63 [b]
Level 2	6.18 ± 0.80	5.83 ± 0.86	6.26 ± 0.56	6.00 ± 0.68 [c]	6.22 ± 0.67	7.33 ± 0.49 [c]	6.93 ± 0.59
Level 3	8.05 ± 0.79	8.17 ± 0.51	8.68 ± 0.75	8.43 ± 0.76	8.11 ± 0.60	9.13 ± 0.52	8.93 ± 0.70
Period	4.23 ± 0.81	4.67 ± 0.69	4.68 ± 0.75	4.29 ± 0.83	4.11 ± 0.33	4.47 ± 0.99	4.33 ± 1.05
Geotaxis							
Level 1	3.77 ± 0.43	3.72 ± 0.46	4.16 ± 0.37	4.14 ± 0.36	4.00 ± 0.50	4.60 ± 0.74	4.53 ± 0.52
Level 2	7.00 ± 0.82	7.06 ± 0.87	7.11 ± 0.74	7.29 ± 0.73	6.67 ± 0.71	7.47 ± 0.52	7.33 ± 0.62
Level 3	9.23 ± 0.81	9.50 ± 0.51	10.0 ± 0.67	10.0 ± 0.68	9.22 ± 0.67	10.0 ± 0.53	9.87 ± 0.64
Period	5.45 ± 0.67	5.78 ± 0.43	5.84 ± 0.76	5.86 ± 0.77	5.22 ± 0.44	5.40 ± 0.74	5.33 ± 0.90
Righting							
Level 1	3.64 ± 0.49	3.67 ± 0.49	3.84 ± 0.37	4.00 ± 0.55	3.78 ± 0.44	4.20 ± 0.56	4.13 ± 0.35
Level 2	6.73 ± 0.77	6.06 ± 0.87	6.42 ± 0.51	6.36 ± 0.50	6.44 ± 0.53	6.80 ± 0.68	6.93 ± 0.70
Level 3	9.05 ± 0.79	8.50 ± 0.62 [a]	8.79 ± 0.71	9.57 ± 0.51 [a]	8.67 ± 0.50	9.40 ± 0.74	9.20 ± 0.41
Period	5.41 ± 0.59	4.83 ± 0.71	4.95 ± 0.85	5.57 ± 0.51	4.89 ± 0.60	5.20 ± 0.86	5.07 ± 0.59
Grasping							
Level 1	8.55 ±0.51	8.44 ±0.62	9.37 ±0.60	9.14 ±0.36	9.11 ±0.33	9.13 ±0.83	9.00 ±0.76
Level 2	9.73 ±0.46	9.72 ±0.75	10.47±0.51	10.86±0.77	10.78±0.44	10.40±0.83	10.13±0.64
Level 3	10.95±0.65	11.39±0.78	11.89±0.57	12.21±0.89	12.44±0.53	11.73±0.70	11.47±0.64
Period	2.41 ±0.50	2.94 ±0.54	2.53 ±0.51	3.07 ±1.00	3.33 ±0.50	2.60 ±0.63	2.47 ±0.52
Bar holding							
Level 1	10.50±0.80	10.56±0.51	10.84±0.37	11.07±1.33	11.22±1.39	10.27±0.80	10.00±0.76
Level 2	13.55±1.10	14.00±0.77	13.05±0.97	13.43±1.02	14.56±1.13	13.20±0.94	13.13±0.92
Level 3	15.95±0.72	16.44±0.86	16.32±0.48	16.57±0.85	17.22±0.67	16.60±0.63	16.33±0.49
Period	5.45 ±0.80	5.89 ±0.96	5.47 ±0.51	5.50 ±1.29	6.00 ±0.87	6.33 ±0.82	6.33 ±0.82

For each test, the first day of occurrence of levels 1, 2 and 3 are shown. Values are means of all pups per group ± SD. The period (difference between level 3 and level 1) shows the time in days that the animals needed to develop a certain behaviour. Scores of groups with identical superscripts per test are significantly different (a, b, c: P<0.05).

No statistically significant differences were found between the treatment groups and the control group in any of the behavioural tests employed. Multiple comparisons revealed significant differences between the various treatment groups for some behaviours: NCh- mice were slower with onset of walking (level 2: $P<0.05$) compared with Ch- mice and Ch+ chimeric mice (walking period: $P<0.05$). In the cliff drop aversion test, B mice started earlier compared with NCh+ and NCh- mice (level 1: $P<0.05$), while Ch+ mice scored significantly faster in level 2 compared with NCh+ mice (level 2: $P<0.05$). Finally, the Ch+ mice were slower with righting compared with the B mice (level 3: $P<0.05$).

***Table V**: Summary of morphological development in mice*

Morphology	**C**	**B**	**BSh**	**Ch+**	**Ch-**	**NCh+**	**NCh-**
Hair growth							
Level 1	6.91 ±0.29	7.06 ±0.24	7.37 ±0.50	7.21 ±0.43	7.00 ±0.00	7.13 ±0.35	7.07 ±0.26
Level 2	8.59 ±0.67	8.67 ±0.49	8.84 ±0.37	8.57 ±0.65	8.56 ±0.53	8.60 ±0.63	8.67 ±0.62
Level 3	9.86 ±0.71	9.83 ±0.51	9.84 ±0.37	10.07 ±0.62	9.67 ±0.50	9.93 ±0.46	10.07 ±0.26
Period	2.95 ±0.65	2.78 ±0.55	2.47 ±0.51	2.86 ±0.36	2.67 ±0.50	2.80 ±0.41	3.00 ±0.00
Ears elevated							
Level 3	4.36 ±0.49	4.11 ±0.32	3.84 ±0.37	4.21 ±0.43[a]	3.67 ±0.50	3.60 ± 0.74	3.40 ±0.51[a]
Ears open							
Level 1	12.86 ±0.47	12.89 ±0.47	12.89 ±0.32	13.00 ±0.68	12.67 ±0.50	12.47 ±0.52	12.33 ±0.49
Level 3	13.95 ±0.38	13.89 ±0.47	14.00 ±0.33	14.00 ±0.68	13.67 ±0.50	13.53 ±0.52	13.33 ±0.49
Period	1.09 ±0.29	1.00 ±0.00	1.11 ±0.32	1.00 ±0.00	1.00 ±0.00	1.07 ±0.26	1.00 ±0.00
Eyes open							
Level 1	13.68 ±0.48	13.44 ±0.51	13.26 ±0.45	13.36 ±0.50	13.11 ±0.60	13.00 ±0.38	13.07 ±0.70
Level 3	14.82 ±0.59	14.67 ±0.84	14.53 ±0.51	14.57 ±0.65	14.44 ±0.53	14.07 ±0.46	14.13 ±0.64
Period	1.14 ±0.35	1.24 ±0.44	1.26 ±0.45	1.02 ±0.43	1.33 ±0.50	1.07 ±0.26	1.07 ±0.26
Lower incisor							
Level 1	9.59 ±0.59	9.28 ±0.46	9.68 ±0.48	9.43 ±0.51	9.78 ±0.67	9.20 ±0.41	9.33 ±0.72
Level 3	10.68 ±0.65	10.28 ±0.46	10.74 ±0.56	10.57 ±0.65	10.78 ±0.67	10.27 ±0.46	10.47 ±0.52
Period	1.09 ±0.29	1.00 ±0.00	1.05 ±0.08	1.14 ±0.36	1.00 ±0.00	1.07 ±0.26	1.13 ±0.35
Upper incisor							
Level 1	10.95 ±0.90	11.44 ±0.51	11.63 ±0.68	11.29 ±1.38	11.44 ±0.73	11.20 ±0.41	11.20 ±0.41
Level 3	12.95 ±1.05	13.39 ±0.50	12.84 ±0.90	13.29 ±0.99	13.00 ±0.87	13.13 ±0.35	13.27 ±0.46
Period	2.00 ±0.82	1.94 ±0.54	1.21 ±0.42	2.00 ±1.18	1.56 ±0.53	1.93 ±0.26	2.07 ±0.59
Nipples							
Level 3	7.89 ±1.36	8.33 ±1.00	7.57 ±0.53	9.00 ±0.58	8.67 ±0.58	9.50 ±0.55	8.83 ±1.17
Milk spot							
Level 3	4.64 ±0.49	4.33 ±0.49	4.05 ±0.23	4.36 ±0.50	4.33 ±0.50	4.87 ±0.35	4.33 ±0.49

For each development, the first day of occurrence of levels 1, 2 and/or 3 are shown. Values are means of all pups per group ± SD. The period (difference between level 3 and level 1) shows the time in days that the animals needed to accomplish a certain development. Groups with identical superscripts per development are significantly different (a: $P<0.05$).

Morphological development

Table V summarises the development of morphological characteristics in the various groups. No statistically significant difference was found between the treatment groups and the control group (C) in any of the morphological development characteristics. Multiple comparisons revealed a significant difference between the various treatment groups only for the ear elevation between Ch+ and NCh- mice ($P<0.05$).

No significant difference in onset of the development of hair growth nor ear- or eye-opening were found between the treatment and control groups. Also there were no differences in the time of the onset of the eruption of incisors. In control C57BL/6 JIcoU mice, inguinal nipples are first shown in females at day 8 or 9. For all groups, the inguinal nipples were visible at the same age as observed in the controls. Milk uptake is visible in the stomach from birth until day 4 or day 5, when the skin is still transparent (level 3). All pups used for testing showed first signs of milk uptake at birth; no significant differences between the treatment groups and the control group of visible milk spots have been found. All pups that died before postnatal day 3 showed little or no milk spots.

Discussion

The present study was performed to investigate the impact of the biotechnology procedures involved in gene targeting, on behavioural and morphological/physiological parameters in mice during their pre-weaning period. To this end, newborn pups, produced by different gene targeting treatments, were compared in their development in various behavioural tests, morphological characteristics and growth, which was recorded daily from birth up to the age of 3 weeks.

Some effects of the biotechnological procedures were found, for example the pregnancy rates of the foster mothers in the Ch+/Ch- group (64.7%, 10/16), which included procedures like the electroporation of ES cells with the gene construct and the subsequent microinjection of ES cells in the blastocyst, were lower compared to the other groups. These pregnancy rates were in the normal range for the ES cell technique at our institute, where normally two out of three foster mothers (66%) used per injection procedure, get pregnant (Hofhuis, personal communication). This implicates that an average of 33% of all fosters mothers is treated without a resulting pregnancy. In contrast, all foster mothers of the NCh+/NCh- group, which included only culture of ES cells (without electroporation) and subsequent microinjection of ES cells in the blastocyst, resulted in a pregnancy rate of 100% (11/11). These results suggest that electroporation might influence the pregnancy rate of the foster mothers.

Furthermore, some adverse effects of the different gene targeting procedures on survival rate were found. After implantation of the blastocysts, which were microinjected with ES cells (with or without the electroporation step), four foster mothers in both Ch+/Ch- and NCh+/NCh- groups experienced perinatal death of their only pup, which occurred before pups were handled for testing. These pups were not cannibalised by their mothers, which regularly

occurs, as pregnant mice are usually very stress susceptible just before and after parturition. The birth of only one pup after transferring 10-12 blastocysts, may also indicate (prenatal) uterine pup mortality. Not considering the above mentioned eight foster mothers, no significant differences in litter size could be detected between the treatment groups and the control group. In all litters used for testing, no significant difference in pup survival was found.

However, the gene targeting techniques did influence the body weight of the offspring. At birth, the average body weight of the treatment groups Ch+/Ch- and NCh+/NCh- was higher than the control C57BL/6 group. During the rest of the pre-weaning period, mice of all treatment groups were heavier than the control C group. The birth of heavier offspring, and higher incidences of perinatal loss, after *in vitro* embryo procedures used before and after the actual microinjection (e.g. embryo culture, embryo transfer, nuclear transfer techniques), has also been reported for cattle and sheep (Walker et al., 1996; Kruip and Den Daas, 1997). When using gene targeting techniques, there is a selection for superior material (the best looking blastocysts are reimplanted), which could contribute to 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.

Early development of sensory and motor responses in mice has been extensively studied employing a battery of tests developed some 30 years ago by Fox (1965). Since then, Fox' tests have proven validity for revealing, often very subtle, individual differences in ontogeny (Crusio and Schmitt, 1996, 1998). It became evident that an individual's genotype plays an important role in the regulation of early development, as has been demonstrated by abnormal development in some neurological mouse mutants (Van Abeelen and Kalkhoven, 1970). In the rodent, cliff avoidance, surface righting and negative geotaxis are behaviours that develop soon after birth (Altman and Sudarshan, 1975; Costa, 1997b). The timing of their appearance and development has been suggested as a parameter for the assessment of the development of the rodent nervous system, and as indicator for the subsequent appearance of more complex behaviours. The neuromotor development of young mice, as assessed in the present study by using a modified version of the Fox' tests during the early postnatal life, only revealed minor effects of the different gene targeting treatments. No developmental retardations were found in any of the treatment groups compared with the controls.

The use of the gene targeting procedures also did not influence the development of morphological characteristics of the resulting offspring, as no difference in the onset of any of these parameters was detected. The presence of a milk spot and the body weight proved easy, and probably important parameters reflecting welfare during the first days of life. All pups dying before postnatal day 3 showed no, or small milk spots, and their body weight was also lower compared to the average body weight of their litter. It is important to observe whether only an individual pup is affected by lack of stomach-filling (possibly transgene-related), or an entire litter, possibly indicating a maternal effect, like absence of milk or poor mothering (Mertens and Rülicke, 1999). Stomach-filling is, therefore, not only useful for individual monitoring, but also gives important clues about specific cause(s) of possible breeding problems (Mertens and Rülicke, 1999).

Conclusion

The impact of the gene targeting technique on the early development of animals has been evaluated using the parameters described in this study. Potential indicators of poor welfare that can be monitored on a routine basis (e.g. by scientists and animal technicians) in the process of producing knockout animals were selected.

Besides a higher body weight and a higher rate of perinatal mortality, 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) have a major impact on the normal development of pre-weaning mice. Nevertheless, the observed effects indicate that gene targeting procedures may affect normal physiology, requiring careful monitoring of health and welfare of the offspring.

Although in the present study only few significant differences were found, it should be stressed that behavioural tests can be very useful for studying early effects of a mutant gene in newly generated knockout mice. Those tests can be employed as routine procedures in the breeding facility, as they are easy to perform, take little time and are non-invasive. Such testing procedures should also include scoring of survival rate, milk spot and body weight of newborn pups at a very early stage, which can be extremely useful for individual welfare monitoring and identifying possible breeding problems.

In the next part of this project the groups of mice monitored in this study were submitted to other behavioural tests, in order to assess different aspects of behaviour, such as locomotor activity, anxiety and exploration of an unfamiliar environment. Also their morphological and physiological development and clinical appearance will be further monitored up until the age of 30 weeks, after which post-mortem examinations will be performed.

Acknowledgements

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Chapter 5

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

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Summary

The effects of gene targeting procedures on the behaviour 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 behaviour 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 behaviour, observed for the two groups of chimeric mice (one derived from electroporated embryonic stem (ES) cells and the other from non-electroporated ES cells) when compared to the other (non-chimeric) 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.

Introduction

Targeted gene mutation technology has resulted in many new knockout or knockin mouse models. These mutant mice have been generated by utilising 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 (Chu et al., 1987; Koller and Smithies, 1992; Wilder and Rizzino, 1993; Bronson and Smithies, 1994). 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 visualised if the ES cells and host blastocyst are derived from mice with different coat colours. 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 behavioural studies have been performed, especially in the areas of neuroscience and psychopharmacology (Wehner et al., 1996; Nelson and Young, 1998; Crawley, 1999; Bolivar et al., 2000). 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 (Crawley et al., 1997).

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 I) in order to determine if the manipulations had any effects on the development or behaviour 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 APC2, a homologue of the adenomatous polyposis coli (APC) tumour suppressor, in development and sporadic colorectal cancer is being studied (Van Es et al., 1999). The APC2 knockout construct has been introduced by electroporation into ES cells, and both the chimeric and non-chimeric 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 behaviour, 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 post-mortem examinations were performed.

Animals and Methods

Animals

Seven groups of mice, all with a different gene targeting background (see Table I), 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 embryonic stem (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 colour. The resulting chimeric mice (Ch+ mice), which have a mixed fur colour 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. Non-chimeric 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).

Non-chimeric 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 summarise, the following groups of animals were used:

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. A total of 22 mice (13 males and 9 females) were tested.

B mice: untreated blastocysts transferred to foster mothers
Blastocysts were isolated, cultured, and transferred to foster mothers without any further gene targeting treatment. A total of 17 progeny mice were available for testing (10 males and 7 females).

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. A total of 19 progeny mice (12 males and 7 females) were available for testing.

Ch+ mice: chimeric animals after electroporation of ES cells with APC2 knockout gene
The APC2 mouse gene construct (kindly provided by van Es et al., 1999) was introduced into the 129/Ola-derived ES cell line by electroporation (250 V, 500 µ, 7.8 ms) using standard procedures (Verbeek et al., 1995). Positive targeted clones were used for microinjection into the blastocysts. Chimeric mice (as identified by mixed coat colour) were obtained by injecting 10-15 gene-targeted ES cells into C57BL/6 blastocysts. An average of 10-12 injected blastocysts were transferred into pseudo-pregnant females (recipient mice). A total of 14 chimeric mice were tested (7 females, 6 males, 1 hermaphrodite).

Ch- mice: non-chimeric animals after electroporation of ES cells with APC2 knockout gene
Non-chimeric animals after procedures were performed for the production of the Ch+ chimeras, as identified by their black coat colour. A total of 9 mice was tested (6 males and 3 females).

NCh+ 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 colour) were obtained by injecting 10-15 ES cells into C57BL/6 blastocysts. An average of 10-12 injected blastocysts were transferred into pseudo-pregnant females (recipient mice). A total of 14 mice was tested (7 males, 6 females, 1 hermaphrodite).

NCh- mice: non-chimeric animals without electroporation of ES cells
Non-chimeric animals after procedures were performed for the production of the NCh+ chimerics (as identified by their black coat colour). A total of 15 mice was available for testing (6 males, 9 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, Hooper et al., 1997; provided by A. Berns of the Netherlands Cancer Institute, Amsterdam) were derived from the 129/Ola (agouti) strain. All foster mothers were B6D2F_1/CrlBR (Charles River, Sulzfeld, Germany). The gene targeting techniques were performed by an experienced technician according to standard procedures

(Verbeek et al., 1995; Shilham et al., 1996; Hazenbos et al., 1996). All animals were tested during the same period of post-weaning development (age 4-30 weeks).

***Table I**: Biotechnological procedures*[a]

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

[a]*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 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 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-=non-chimeric).*

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^2; Tecniplast, Rome, Italy) with sawdust bedding (Pine-wood ¾; Woodyclean, BMI, Helmond, The Netherlands). Per cage, a tissue (Kleenex, Kimberly-Clark Corporation, 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 hours light: 12 hours dark cycle with lights on from 0600 hours to 1800 hours, 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*.

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.

Behavioural tests

During the 6 months of study, animals were subjected individually to several behavioural tests from weaning onwards. The different groups were tested in a randomised manner between 1500 and 1700 hours. The tests, which were used to compare the development of the different groups, have been previously described (Van der Meer et al., 2001a). In short:

Hole board test

Exploratory behaviour was studied in a square 16–hole (Ø 3 cm) board task, measuring 37.5x37.5x3.5 cm, covered with a transparent Perspex lid (40x40x20 cm) (Boissier and Simon, 1962; Van de Weerd et al., 1994). 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, grooming, and faeces and urine production were also registered for each mouse.

Cage emergence test

A mouse is placed in an unfamiliar cage (Macrolon Type I cage, size 24x13.5x13 cm, with a ∅ 4 cm hole in one sidewall, no lid on top), with its head opposite of the opening (Van de Weerd et al., 1994). 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.

Behavioural profile as registered by LABORAS™

The automated behaviour registration system LABORAS™ (Laboratory Animal Behaviour Observation, Registration and Analysis System; Metris, Hoofddorp, The Netherlands), validated by Van de Weerd et al. (2001), has been described in detail (Bulthuis et al., 1997; Schlingmann et al., 1998). With this system, the positions and six behavioural categories: 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 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. Mice were tested at the age of 22-24 weeks.

During 24 h, the behaviour 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 behavioural category was calculated and analysed.

Directly after the 24-h test, a metal climbing grid (size 16x10 cm, mesh size 0.5x0.5 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 behaviour. The relative mean time spent on climbing behaviour 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 behaviour test for the same animals.

Light-dark test

Anxiety-related behaviour was investigated in a light-dark test (Lister, 1990) by using a cage adapted (Van der Meer et al., 2000) for LABORAS (Macrolon Type III, 38x22x27 cm, two equally sized compartments, one illuminated by 1000 lx). A clear Perspex tunnel (10x6x5 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).

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 1600 and 1700 hours. The behavioural response during marking of the tail was scored (ranging from 1 to 7; Van de Weerd et al., 1997), as well as several other responses of the animals (biting, freezing, or urine/faeces production) during the manipulation. Subsequently, the behaviour of the animals after handling was observed in their home cage for 10 min using a sampling method, wherein every 5 s the behaviour of the animal was scored according to a predefined ethogram based upon Blom et al. (1992). The following behaviours are distinguished: immobility, locomotion, rearing, grooming, digging, climbing, eating, social behaviour, and fighting.

Post-mortem examinations

At the end of this study, animals were killed and post-mortem 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.

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 behavioural measurements, repeated-measurements ANOVA was also used for the hole board test, the (LABORAS) 24-h behaviour 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 behavioural 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. Behaviour performed by mice during the hole board test, the cage emergence test, and the handling test was analysed using non-parametric statistics, i.e., the Kruskal-Wallis test followed, where appropriate, by nonparametric multiple

comparisons tests (Siegel and Castellan Jr., 1988). The level of statistical significance was pre-set at $P<0.05$ 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.

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

Results

Survival rate

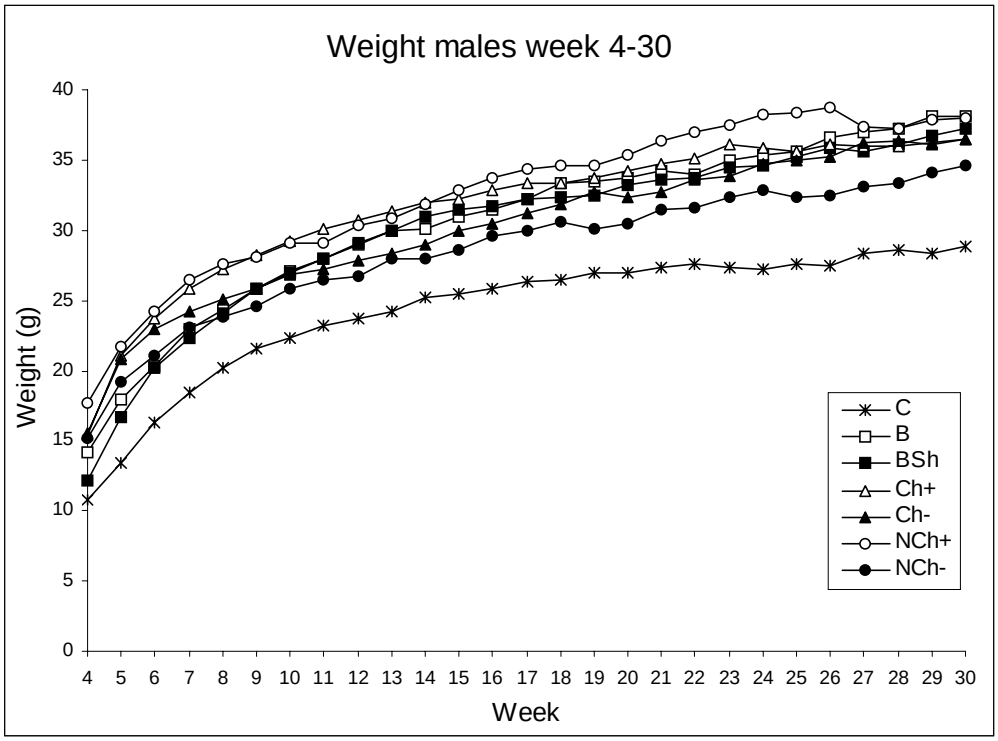
No major effects of the different gene targeting treatments were found on survival rates. Only one B female died with unknown cause at week 6.

Body weight/growth rate

Figure 1 shows the average body weights of the mice during the experiment. Repeated-measures ANOVA revealed significant effects for group ($F(6,94)=14.78$, $P<0.001$) and gender ($F(1,94)=93.30$, $P<0.001$) 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 post-weaning period ($P<0.001$), but no differences in growth rate (weight gain per week) were detected. The heaviest mice were the chimeric NCh+ males and Ch+ females. Multiple comparisons demonstrated no significant differences in body weight between the other groups. Overall, males weighed significantly more than females ($P<0.001$) for all groups.

Exploratory behaviour

Repeated-measures ANOVA revealed a significant effect of group on the number of holes explored in 3 min ($F(6,94)=9.86$, $P<0.001$), while no effects for gender were detected. Multiple comparisons demonstrated that chimeric Ch+ mice expressed significantly ($P<0.01$, Figure 2) less exploratory activity than control (C) mice and B mice in both tests. In addition, the chimeric NCh+ mice displayed significantly ($P<0.05$) 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 ($F(1,94)=24.01$, $P<0.001$, 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 ($P<0.01$), while the faeces production of Ch+ and NCh+ mice was significantly higher than the control group ($P<0.01$). Overall, males produced significantly more faeces and urine and performed more grooming behaviour than females for all the groups ($P<0.01$).



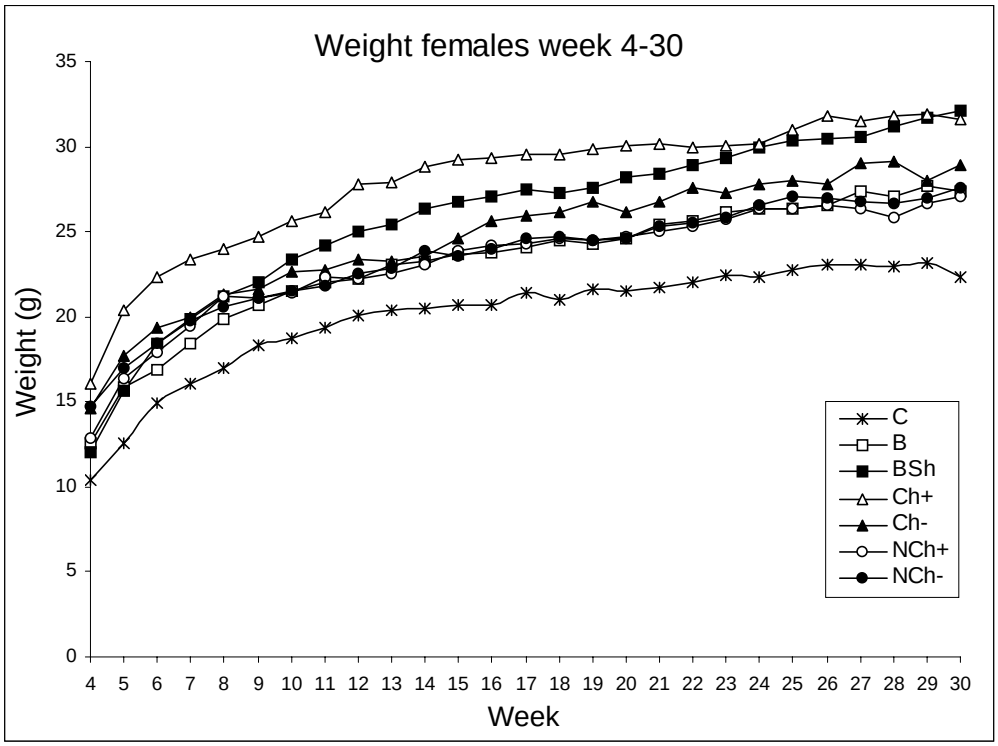


Figure 1: *Mean male (Fig 1a) and female (Fig 1b) body weight (g) in post-weaning period (age 4-30 weeks).*

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+: Chimeric animals after electroporation with APC 2 knockout gene (B6/129)
Ch-: Non-chimeric animals after electroporation with APC 2 knockout gene (B6)
NCh+: Chimeric animals without electroporation (B6/129)
NCh-: Non-chimeric animals without electroporation (B6)

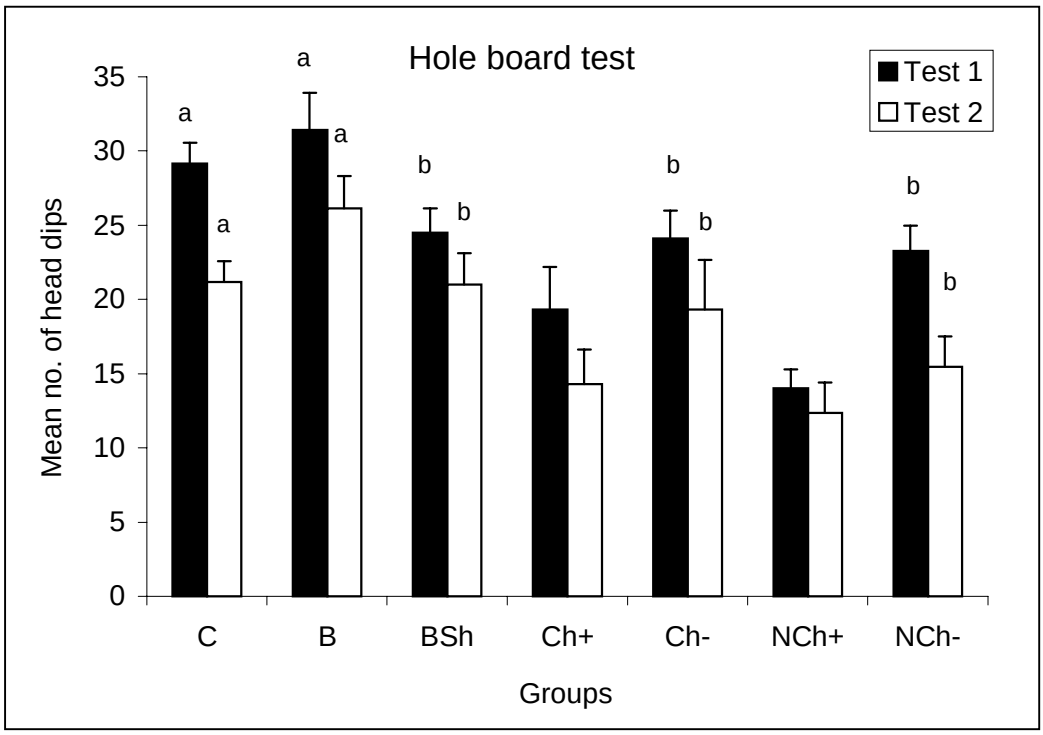


Figure 2: *Exploratory behaviour in two subsequent hole board tests.*
Testing was performed at the age of 12 weeks (Test 1) and 14 weeks (Test 2) between 1500 and 1700 h. Test period was 3 min. Data are expressed as mean numbers of head dips ± S.E.M. The chimeric Ch+ and NCh+ mice were less explorative compared to the other groups in both tests. ($^{a}P<0.01$, significant difference compared to both Ch+ and NCh+ groups, $^{b}P<0.05$, significant difference compared to NCh+ group only).

Cage emergence test

There was a significant effect of group on the time to escape from an empty cage ($F(6,101)=3.58$, $P<0.01$). Multiple comparisons demonstrated that chimeric Ch+ mice needed significantly more time to escape (Figure 3) compared to the control C mice ($P<0.01$), B mice ($P<0.05$), and NCh- mice ($P<0.05$). All animals left the cage within the maximum time set for the test (10 minutes). Most animals left the cage within 60 seconds. However, Ch+, NCh+, and BSh mice showed greater variation in time to escape when compared to the other groups ($P<0.05$). No differences between males and females were found between groups. Also, no significant differences between groups were found for the various behaviours scored during the test. The behaviours most frequently observed were rearing to the sidewalls and sniffing at the hole.

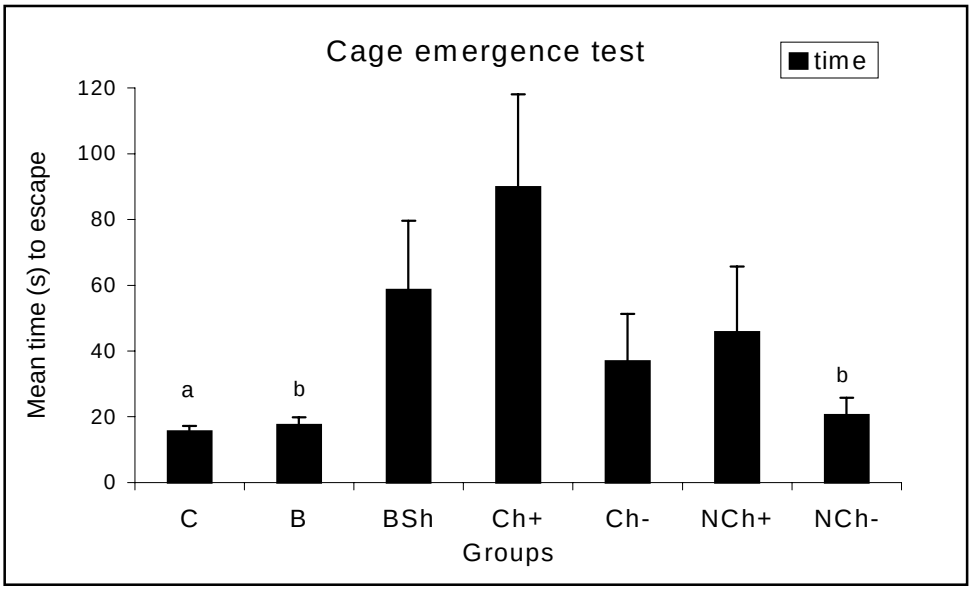


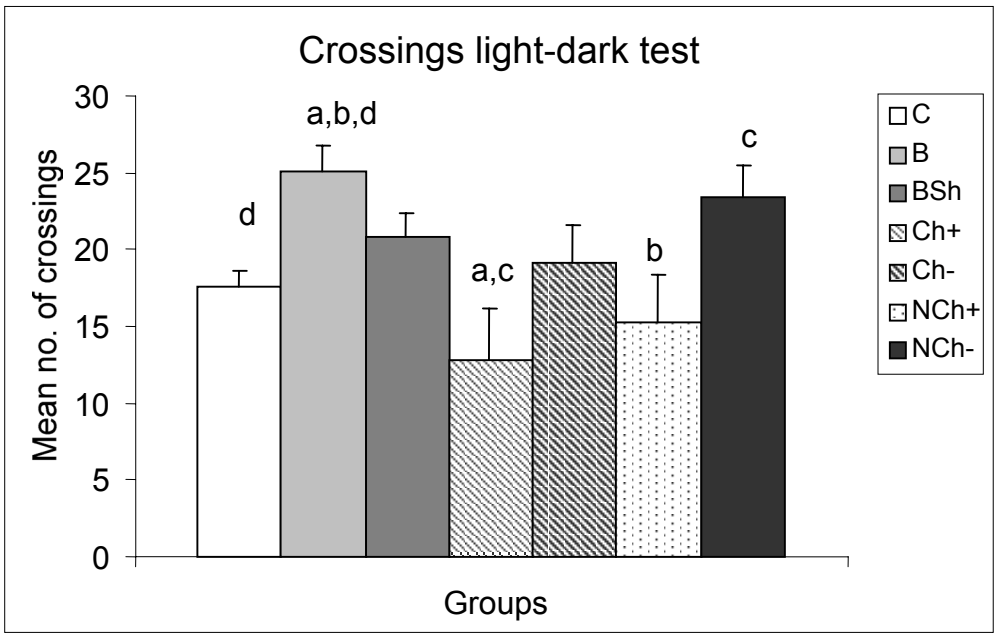
Figure 3: *Mean time (s ± S.E.M.) to escape from an empty cage.*
Testing was performed at the age of 16 weeks between 1500 and 1700 h. Maximum testing period was 10 min. Differences in reactivity were found for chimeric Ch+ mice compared to some other groups. ($^{a}P<0.01$, $^{b}P<0.05$, significant difference compared to Ch+ group).

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 Figure 4a and b). Gender effects were only observed for the latency.

Overall, there was a significant effect of group for the number of crossings ($F(6,91)=4.21$, $P<0.01$). Multiple comparisons demonstrated that chimeric Ch+ and NCh+ mice showed significantly less crossings (see Figure 4a) compared to the B mice ($P<0.05$). The Ch+ mice also showed fewer crossings compared to the NCh- animals ($P<0.05$).
In addition, the B mice displayed significantly more crossings than the controls ($P<0.05$). 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 (Figure 4b). However, both chimeric Ch+ and NCh+ mice showed greater variation in time until their first entry compared to the other groups ($P<0.01$). For all groups, effects in males and females were similar, although overall males showed increased latency compared to females (($F(2,91)=3.71$, $P<0.05$; overall mean males 131.4 sec. vs. mean females 96.3 sec.). Mice from all groups showed a preference for the dark compartment, as measured by total time spent in the dark versus light during the 10 min test sessions. Overall, there was a significant effect of group for time in the light compartment ($F(6,91)=4.29$, $P<0.01$). Total time spent in the light is significantly shorter for Ch+ and NCh+ mice compared to the control group ($P<0.05$) and the non-chimeric Ch- and NCh- groups ($P<0.01$).



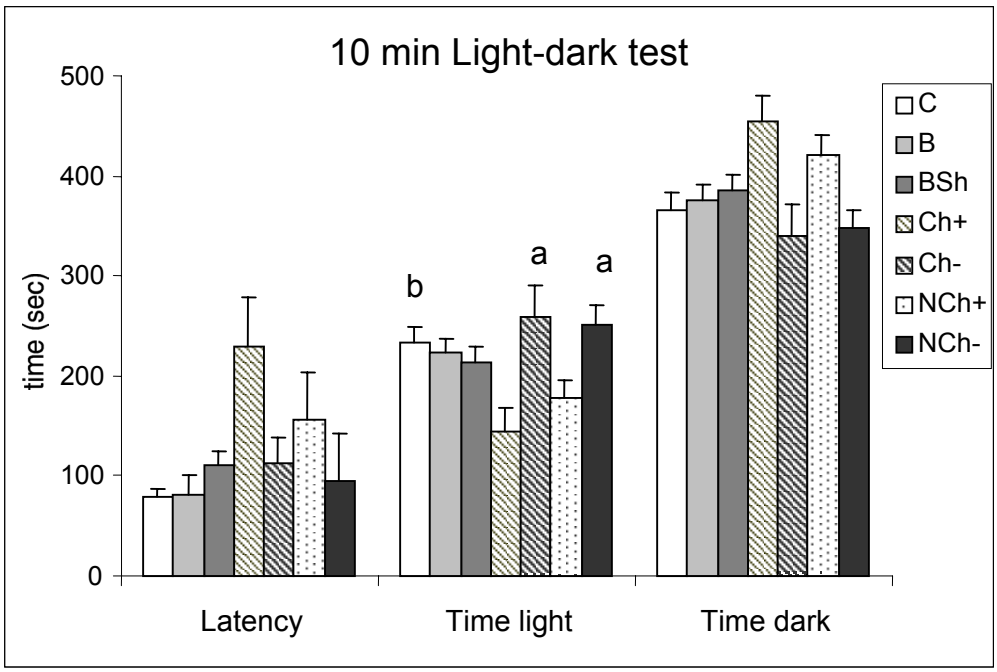


Figure 4: *Results of the light-dark preference test. (a) Mean number of crossings between the two compartments, (b) Light-dark test, start from dark compartment.*
Number of crossings between the two compartments (a), latency to first entry (b), and total time spent in the light (b) and in the dark (b) compartment (in sec) of the light-dark test (mean ± 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 1500 and 1700 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, $P<0.05$; (b) $^{a}P<0.01$; $^{b}P<0.05$, significant difference compared to both Ch+ and NCh+ groups).

LABORAS 24-h test

Figure 5 presents the results of the 24-h behaviour observation as recorded by LABORAS. Per time period, the relative mean time spent on each of the six different behaviour 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 went on again in the 13-15 h period. Gender effects were present for climbing and eating behaviour.

Immobility (Figure 5a)

Overall, there was a significant group effect for immobility ($F(6,90)=2.91$, $P<0.05$). During the 1-3 h, 4-6 h, and 7-9 h periods, Ch+ mice showed more immobility compared to the controls ($P<0.01$), and all other groups ($P<0.05$), 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 ($P<0.05$). During the light period, no significant differences in duration of immobility were found between the groups.

Locomotion (Figure 5b)

Overall, ANOVA revealed a significant group effect for locomotion ($F(6,90)=4.86$, $P<0.001$). The Ch+ mice showed significantly less locomotion compared to control group C ($P<0.01$) and all other groups ($P<0.05$), 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 ($P<0.1$).

Climbing (Figure 5c)

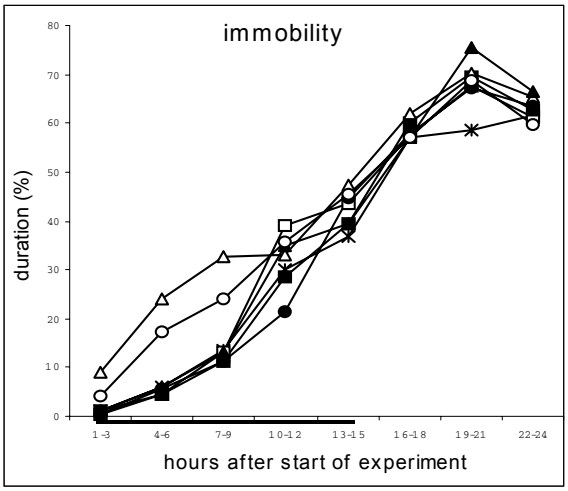
Overall, the chimeric Ch+ and NCh+ mice spent significantly less time on climbing compared to the non-chimeric NCh- mice (ANOVA group effect: $F(6,90)=4.98$, $P<0.001$; 1-24h, Ch+ and NCh+ vs. NCh-: $P<0.01$). These differences were mainly caused by their climbing behaviour during the first three periods (dark period). No significant differences between the other groups were found. Overall, females spent more time on climbing than males for all groups ($F(1,90)=16.22$, $P<0.001$; mean 10.36% vs. 6.64%).

Grooming (Figure 5d)

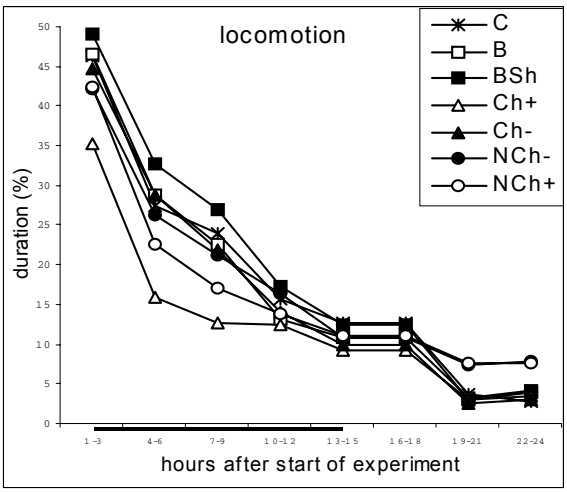
No significant differences were found in grooming behaviour for all groups. Overall, the mean percentage of time spent on grooming was 13%.

Drinking (Figure 5e)

For the 1-3 h, 4-6 h, and 7-9 h periods, the B and BSh- mice showed significantly less drinking behaviour compared to the controls (ANOVA group effect: $F(6,90)=2.25$, $P<0.05$; B and BSh- vs. controls: $P<0.05$).



5a



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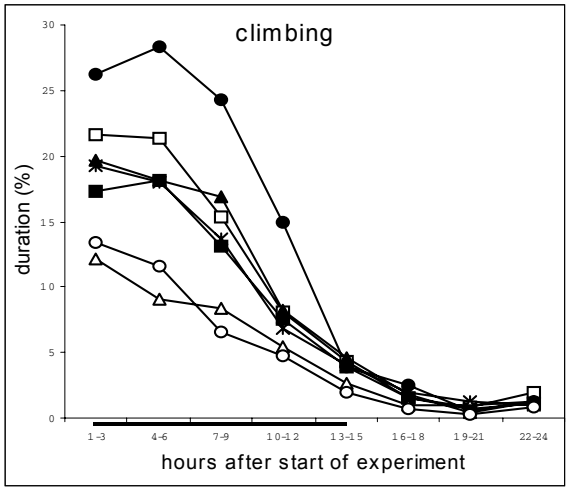

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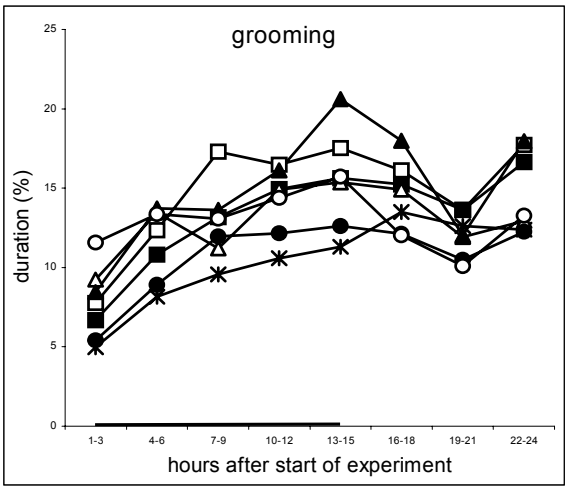

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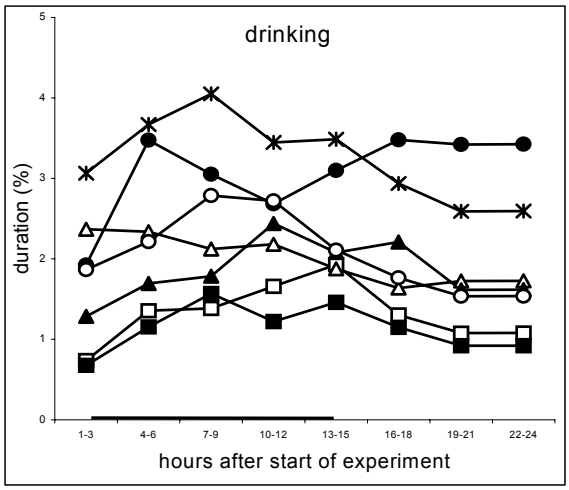

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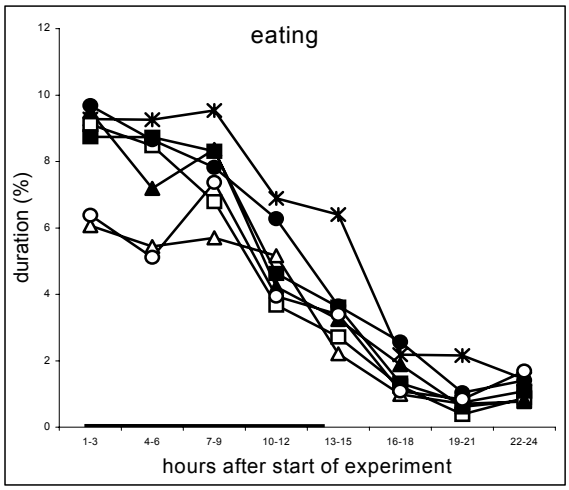

5f

Figure 5: *Results of the LABORAS behaviour registration system, 24 h test.*
Per time period of 3 h, the relative mean time spent on each of the six behavioural 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.

Eating (Figure 5f)

The Ch+ and NCh+ mice showed significantly reduced eating behaviour when compared to the controls during the first two periods (ANOVA group effect: $F(6,90)=2.79$, $P<0.05$; Ch+ and NCh+ vs. controls: $P<0.05$). For all groups, effects for males and females were similar, although overall females showed more eating behaviour compared to males ($F(1,90)=7.24$, $P<0.01$, overall mean females 5.2% vs. mean males 4.1%).

LABORAS 12 hours extra climbing

Directly after the 24-h test, the animals were tested for extra climbing behaviour by adding an extra climbing grid to the cage. During the following 12 h (dark period), the climbing behaviour was recorded. Overall, females spent more time on climbing than males for all groups ($F(6,31)=2.52$; $P<0.05$). This difference is less significant for the Ch+ and NCh+ chimeric mice, where both males and females showed less climbing behaviour (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.

***Table II**:*

Absolute organ weights expressed as mean (± S.E.M.) grams x10^{-2}, split by group and gender

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

Values are means of 6 males or 6 females per group (± S.E.M., in grams x 10^{-2}), except for Ch- mice (3 females). Body weights are shown in grams. ([a]P<0.05, [b]P<0.01 significant difference compared to control C mice).

Handling test

The score of the response to handling and the behaviour after handling scored for 10 minutes in the animals' home cage did not reveal any overall significant difference between the groups (results not shown).

Post-mortem 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 post-mortem examination ($F_{males}(6,34)=3.64$, $P<0.01$; ($F_{females}(6,39)=6.26$, $P<0.001$; Table II, see also Figure 1). Males of all the treatment groups and BSh, Ch+, and NCh- females were all heavier than the controls ($P<0.05$). 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). 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: $F(6,34)=3.01$, $P<0.01$; Ch+ and NCh+ vs. controls: $P<0.01$) and Ch+ and BSh females (ANOVA group effect: $F(6,39)= 3.83$, $P<0.01$; Ch+ and BSh vs. controls: $P<0.05$) 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 $F(6,34)=3.51$, $P<0.01$; B and Ch- vs. controls: $P<0.05$), while this was also apparent for the BSh, Ch+ and NCh- females (ANOVA group effect: $F(6,39)=8.15$, $P<0.001$; BSh, Ch+ and NCh- vs. controls: $P<0.05$). 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: $F(6,34)=3.39$, $P<0.05$; B vs. BSh-: $P<0.05$) due to some extreme fat livers of B males.

Post-mortem 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 an ovary (4x2 mm), oviduct, and uterus on one side and a small testicle (3x2x2 mm) and spermaduct 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 epididymes, 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 also 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.

Discussion

The present study was performed to investigate the impact of the biotechnology procedures, involved in gene targeting, on behavioural and physiological parameters in mice during their post-weaning development.

At weaning, the average body weight of all treatment groups was higher than the control C57BL/6 mice. During the whole post-weaning 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 (Kruip and Den Daas, 1997), 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 non-chimeric groups (B; BSh; Ch- and NCh-).

By using behavioural 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 behaviour 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 (Van der Meer et al., 2001a). The animals were less active and more hesitant, sniffed more, and walked less deliberately, which might indicate that explorative behaviour is diminished due to habituation with time or reduced curiosity (Van de Weerd et al., 1994; Dorr et al., 1971). 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+ mice only. The chimeric NCh+ mice showed a greater variation in time to escape from the novel environment. Although all animals escaped from the cage within 10 minutes, and the majority even within 60 seconds, more Ch+ and NCh+ mice escaped after these 60 seconds than in the other groups. This could indicate enhanced anxiety (Lister, 1990).

The light-dark test has frequently been used to test anxiolytic properties of new drugs (File, 1992). 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 behaviour observations showed similar behavioural 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 behavioural patterns are consistent with circadian rhythms of mice as found by others (Schlingmann et al., 1998; Van de Weerd et al., 2001). The chimeric Ch+ and NCh+ 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 (Baumans et al., 2001). Adding an extra climbing object to the LABORAS cage as environmental enrichment did not result in increased climbing behaviour 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 on and thus were used to the handling routines. Also, the chimeric Ch+ and NCh+ mice showed no increased (stress) response to handling.

The fact that no differences in behaviour between the control group and the four groups of non-chimeric animals were found indicates that the biotechnological procedures have no impact on the behaviours as tested in this study. Therefore, the behavioural differences as found between chimeric and non-chimeric 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 behaviour of both strains (Gerlai, 1996; Crawley et al., 1997; Gingrich and Hen, 2000). Previous studies have shown that the two inbred strains display marked differences in performance in a variety of behavioural tasks (Montkowski et al., 1997; Rogers et al., 1999; Kopp et al., 1999): the B6 strain is more explorative and less anxious than the 129 strain. Therefore, the observed differences in behaviour between the chimeric and non-chimeric 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 post-mortem 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 (Shomer et al., 1997). Although the occurrence of hermaphroditism in chimeric animals has previously been reported (Jankowska-Steifer et al., 1992; Shomer et al., 1997), 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 post-mortem examinations may reveal that the welfare of these animals is compromised more severely than can be concluded from the behavioural 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.

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 behavioural differences were most apparent in chimeric (Ch+ and NCh+) 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 behavioural tests, it might be concluded that chimerism does not substantially decrease the welfare of these animals. However, since post-mortem 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, post-mortem examinations of both chimeric and non-chimeric animals is applied on a routine basis, in order to further evaluate the consequences of chimerism for the well-being of the animals.

Acknowledgements

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Chapter 6

Use of score sheets for welfare assessment of transgenic mice

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Summary

The use of transgenic mice has increased dramatically in recent years and continues to increase further. However, because transgenesis may alter a balanced genotype and produce unpredictable effects, careful monitoring of health and welfare of the transgenic animal is requested. The present study assessed the feasibility of the use of score sheets for monitoring transgenic mice, as part of daily routine, in a transgenic unit. The score sheets used were based on sensitive and easy to determine parameters. The score sheets were used by two animal technicians and a thorough evaluation showed that the score sheets, as described in this paper, are useful for routine monitoring in a transgenic unit and may result in the early detection of animal welfare problems. However, notwithstanding the limited number of parameters included and the restricted age-span covered by the screening, the monitoring system was considered time consuming. Large-scale implementation of such a scoring system during the first weeks of age, would increase daily care time with at least 15-20 min for an average litter of 4-6 pups. Nevertheless, the use of score sheets seems a prerequisite for monitoring the animal's welfare in the course of producing transgenic lines.

Introduction

The development of transgenic and targeted mutant (knockout) technologies over the past decade, led to a rapid increase in the number and varieties of genetically modified mice. However, the effects of genetic manipulation are still incompletely understood and the characteristics of the transgenic animals are frequently found to differ from those anticipated. This is caused by limitations in control of the insertion of the DNA, which are inherent in some techniques (e.g. microinjection). In other situations it is attributed to the unexpected interaction of the introduced DNA with other genes. These interactions vary with the genetic background, as has frequently been observed with mice (Gordon, 1997). Thus, transgenic technology can alter a balanced genotype and produce unpredictable and unexpected effects. Interfering with the genotype by inserting or removing fragments of DNA may result in a drastic alteration of the animal's normal genetic homeostasis which can be manifested in the behaviour and well-being of the animals in unpredictable ways (Costa, 1997a; Cockayne et al., 1994; Ting et al., 1994). Uncontrolled expression of inserted genes may result in an increase in morbidity and mortality, frequently a problem encountered using microinjection of multiple copies of a gene and the resulting overexpression or overproduction of the gene product (Mepham et al., 1998). Replacement or disruption of functional genes with non-functional counterparts (targeted mutagenesis/ knockouts) results in failure to produce a functional gene product. This technique has been applied in mice to create models of human and animal pathology or disease. In situations where pathology is established, it is likely that animals suffer to some degree (Mepham et al., 1998).

The unpredictable and uncontrollable nature of the production techniques used for making transgenic mice indicate a need for more care for the animals after the production has been completed to ensure a reasonable quality of life. Animal care-givers are now faced with the difficult task of developing general guidelines to assess and ensure the welfare of these animals. Despite the relatively long history of the science of genetic manipulation, there is, at the moment, no European legislation specifically concerning transgenic animals during production and use. There are some directives, which cover their use, for example, Directive 86/609/EEC regulating the use of animals in experiments and testing, and the directives on genetically modified organisms (GMOs). The implementation of these directives on GMOs within national laws is left to each individual EU-Member. This means that the extent of protection and care for transgenic animals varies with the EU-Member. For example, some countries may define continued breeding of an established transgenic strain as an experiment, whereas other countries may not, leaving the welfare of future generations of mice unprotected.

In the Netherlands, the generation and maintenance of a transgenic strain requires approval from the Committee on Animal Biotechnology on top of the regular approval by local Animal Care and Use Committees. Also, the Inspectorate demands that researchers keep an "animal welfare assessment diary" for every transgenic strain they use. However, the definite content of this diary has not been specified (Code of Practice, 2000). Any welfare assessment of transgenic mice should not only include identification of "intended" adverse

effects (as for animal models of disease), but also the general monitoring of parameters that may be considered as indicators of the well-being of the animals. The unpredictable nature of non-intended effects such as those caused by insertional mutations and genes of unknown or hypothetical function may then also be covered. This welfare assessment scoring needs not only to be repeated for every single newly produced strain of transgenic animals, but it should also be a continuous monitoring, as not all effects of the transgene will necessarily be apparent immediately. For the welfare assessment to be useful on a routine basis it should lead to an "early as possible" detection of impaired well-being. When the assessment indicates that welfare is impaired, measures should be taken to alleviate the suffering, for example, feeding regimen or housing situation could be changed. In this way, the group of animals monitored itself will directly benefit, as well as future generations of transgenic mice.
Many criteria for the quality of welfare of animals have already been put forward (Broom, 1986, 1991; Morton and Griffiths, 1985; Morton, 1997; Barnard and Hurst, 1996; Rowan, 1997), including physiological effects (e.g. growth, reproduction, longevity, immune suppression, corticosteroid levels, disease, injury) and behavioural responses (e.g. preferences, stereotypies, anxiety). However, it is impractical to include them all in any program of routine monitoring because of time and labour related constraints. Therefore, the challenge is to select measurable biological parameters that will cover most of these criteria, while enabling the monitoring of a large number of animals. Recently, a number of methods have been explored for monitoring the health and welfare of transgenic mice using score sheets (Morton, 1998; MUAWC, 1999; Mertens and Rülicke, 1999, 2000). In those cases the scoring has been (or will be) performed by researchers on a separate experimental basis and is not incorporated into the existing routines of animal care, or is involving a very large number of parameters.

We have developed a scoring system, containing a limited number of sensitive, easy to determine and non-invasive parameters, selected from our previous studies on implications of transgenesis for the well-being of mice (Van der Meer et al., 1999, 2001a,b,c). This scoring system has been tested, to assess the use of the score sheets for monitoring the welfare of transgenic mice on a practical basis, as part of the animal technicians' daily routine in a transgenic unit. The score sheets include both the pre- and post-weaning period of the mice. Screening mice from birth allows any innate deficiencies to be identified and quantified at an early stage of life, rather than waiting for such effects to become manifest at a later stage (Costa, 1997a,b). Days 0 to 6, 10 and 14 were selected based on expected developmental progress (Baumans, 1999; Van der Meer et al., 1999, 2001b). Up to 14 days the monitoring used mainly developmental parameters provided by a guideline which features and behaviours are expected in normal developing mice. The score sheets from weaning onwards included more behavioural parameters (Van der Meer, 2001a).

Study design

Three score sheets were developed, two for the pre-weaning period (sheets A and B) and one for the weaning and post-weaning period (sheet C). The score sheets were aimed at quick and easy scoring, meaning the number of tests involving individual handling procedures was kept to a minimum. The score sheets required mainly yes/no answers. There were no invasive techniques included; only observations and basic handling. The monitoring lasted from birth up to 5 weeks of age. Two animal technicians of the Central Laboratory Animal Institute, Utrecht University, performed the scoring. Fifteen B6D2F_1/CrlBR (Charles River, Sulzfeld, Germany) foster mothers were used as recipients for the genetically manipulated egg cells. Monitoring of 64 pups from three newly produced transgenic strains occurred on the following developmental stages: days 0 through 6, day 10, day 14, at weaning (week 3), one week after weaning (week 4) and two weeks after weaning (week 5). The first 6 days after birth, the animals were not scored individually (to avoid possible cannibalism by the foster mother due to disturbance of the nest). The scoring was performed on survival and food intake (milk spot) by indicating how many pups qualified for each category (score sheet A). It was not necessary to remove the pups from the cage during monitoring. The next stage of development was monitored on day 10 (score sheet B). The pups were now individually marked and scored on survival, morphological and sensorimotor development. Scoring on day 14 was similar to day 10, but 2 extra parameters were added, namely eyes and ears open or closed. The same score sheets were used at weaning as well as for post-weaning, at 4 and 5 weeks of age. These sheets are slightly more complicated, as there are frequently more options available than just yes or no. The animals were scored on survival and developmental factors, and also on behavioural characteristics and/or abnormalities and signs of ill health (score sheet C).

The objectivity of the test was assessed by scoring some of the same litters (n=5) by both technicians. In addition, during data collection, the technicians provided a verbal evaluation of the score sheets and filled out a multiple choice evaluation form afterwards. This consisted of four parts containing several questions such as time (estimation of time needed, possibility to implement the procedure in the daily routine), clarity (of score sheets and observations), disturbance (of mother and pups) and general comments/criticism (opinion on the use of such scoring sheets, parameters missing).

Results and Discussion

The main goal of the study was to examine the general feasibility and usefulness of the score sheets and to determine whether animal technicians were able to perform the monitoring, as part of the daily routine.

Time element

The evaluation form showed that completing the score sheets was time consuming and could not generally be incorporated into the existing work routine in the transgenic unit, as there were approximately 4000 mice present at the time. However, the animal technicians agreed that welfare monitoring of transgenic mice should be performed and that they are the persons best qualified for this job. It seems likely that the time required will be less, when the scorers become more experienced. However, monitoring should still be considered an additional task on top of the regular workload. As such, extra time and funds should be made available if it is to be implemented successfully.

Use of the score sheets

Throughout the monitoring procedure, the animal technicians filled in the score sheets independently and with no extra guidance. They considered the forms to be clear and concise, and all the observations and tests easy to perform. The technicians would have liked to see more possible answers on the score sheets than just yes (+) or no (-). It is recommended to extend the range of answers with +/- for the developmental parameters of sheet B (fur; nipples; incisors). Further differentiation, however, could increase the subjectivity and is more time consuming. A high level of objectivity is important, as the aim is to create score sheets that can be filled in by a variety of scorers. For practical use as part of the daily routine, it is important that more technicians are qualified for the monitoring, especially in large laboratories with varying working schedules. It is difficult though, particularly on behavioural parameters, to avoid personal interpretation completely. When both technicians scored the same litters independently, all parameters were scored identical, except for some differences for the scores on reaction to handling and cage opening (sheet C). Although some variation can be expected, it is our feeling that, in case more technicians are involved in the scoring, some instructions in advance on how to interpret a given observation may prove beneficial in reducing the individual variation among scorers. Although it can be expected that experienced technicians may recognise many of the signs depicted on the score sheets, the scoring system presented is also very useful for training newer and less experienced research staff and animal technicians, as it will help them in how and what to observe in the animals.

In order to save time it was recommended to present the score sheets in the form of a log-book with a front cover sheet, where previous scoring days can be seen. The time consuming job of filling in general information could then be cut to a minimum. On the current sheets date of birth, DNA construct, name of scorer and foster mother had to be filled in repeatedly, on every score sheet.

Different score sheets were used, during both the pre- and post-weaning period. Monitoring of pups on days 0 through 6 was not very time-consuming (<5 min. for one average litter of 4-6 pups) and the disturbance levels for both foster mothers and pups were considered acceptable. However, the strain of the (foster) mother used is important. The foster mothers monitored here ($B6D2F_1$) are rather docile compared to some of the other strains used. In more nervous strains, disturbing newly born litters of pups could result in cannibalism or neglecting of the pups by the mother. Also when monitoring further breeding

of transgenics, aspects like the strains of females and the use of primi- or multiparous females (Baumans, 1999) are points for consideration. It was possible to incorporate the initial scoring into the daily schedule, as new litters were routinely checked for number of pups and pup viability already. Day 10 and 14 scoring took some more time as the mice had to be individually marked (approx. 5-10 min for one average litter of 4-6 pups), but it could also be incorporated into the schedule.

Traditionally, weaning is a disturbing activity for young mice, especially when their tail tip has to be removed for DNA detection and an ear clip has to be performed for identification purposes. It was thought that the extra tests involved in monitoring welfare at weaning did not increase the disturbance or discomfort level. It did, however, increase the amount of time spent on the weaning procedure, especially when large litters were involved (approx. 10-15 min. for one average litter of 4-6 pups). Testing of the animals routinely after weaning involves a lot of extra work (approx. 15-20 min/litter) for the animal technicians and this cannot readily be incorporated into the existing daily schedule. Usually, the mice are only observed superficially when the cages are cleaned. Now, every animal had to be examined and weighed individually. The weighing in particular was time-consuming. However, depressed weight gain in young animals or abnormal weight loss in adults is an extremely useful indicator of poor welfare (Broom, 1993). Therefore, although it is time-consuming and maybe stressful to the animal (each mouse must be individually handled), it is our feeling that this aspect cannot be abandoned. Furthermore, the handling itself has an important function as it draws the technicians' attention to any other problems that are not specifically mentioned on the score sheet and might be missed otherwise, like hypothermia or the presence of tumours. Moreover, the animal's reaction to the handling is also an important behavioural parameter. Information on the expected phenotype of transgenic mice should be available to the animal technician, in order to be able to monitor the animals more carefully.

Parameters used

From the evaluation form it was shown that the animal technicians did not feel that any parameters were missing on the score sheets. There were some suggestions for changes concerning the make-up of the current score sheets. For example, they felt some parameters could be left out. Whisker chewing is not very common and thus could be discarded on the general form and added as a comment when it occurs. The same goes for cannibalism. It is difficult though where to draw the line. On the one hand the observations must be simple, quick and non-invasive but on the other hand they must be effective and provide information that is relevant for animal welfare considerations. There must also be sufficient different parameters so as to make statements concerning welfare possible. A single indicator could show that welfare is poor, but absence of an effect on an indicator does not necessarily prove that welfare is good (Broom, 1993). By leaving certain parameters out it is possible that these factors will be overlooked on the few occasions that they occur. The same argument holds for the possibility of having the technicians only fill in the answers that are "unusual" or "unexpected". This would most likely significantly lessen the amount of time spent on monitoring. It would, however, also make it impossible to tell whether monitoring had taken

place at all and will most likely lead to less precise work as the mice would not be routinely screened. Furthermore, it would rely strongly on personal observations, as the scorers would have to note "something is wrong". This would defeat the purpose of routine monitoring, as it is the idea that routine monitoring will effectively take over the job of identifying problems.

It is of utmost importance that the score sheets are simple to minimise the time needed to complete them (see also Lloyd and Wolfensohn, 1999; Lloyd et al., 2000). By introducing more parameters and/or more possible answers the sheets would become more complicated and the time needed to complete them would increase. Further research to determine the functionality of the score sheets should help decide whether extra measurements and observations are actually necessary.

The score of the milk spot proved to be particularly predictable as an indicator of pup survival. All pups with a visible milk spot throughout the first week survived, whereas pups not meeting this criterion died. It is important to observe whether only an individual pup is affected by lack of stomach-filling (possibly transgene-related), or an entire litter, indicating a putative maternal effect, like absence of milk or poor mothering (Mertens and Rülicke, 1999; Lloyd et al., 2000; Van der Meer et al., 2001b). Therefore, stomach-filling is not only useful for individual monitoring, but also gives important clues about specific cause(s) of possible breeding problems (Mertens and Rülicke, 1999).

Further monitoring

The score sheets described in this study proved to be feasible for routine monitoring in a transgenic unit, provided that the additional time needed for the observations, funds and well-instructed technicians are made available. However, there are some points that would further increase the value of the monitoring system. The present study included only the first five weeks of age of newly produced transgenic mice. Score sheets are also useful for monitoring the further breeding of transgenic lines. This monitoring should last from birth until either spontaneous death of the animal or death caused by euthanasia (at the end of the experiment), for at least two generations (Broom, 1997). Elements like mating behaviour, gestation, rearing of the young, milk production and the number of pups from the next generation that reach weaning age could shed a light on the reproductive success of the transgenic mother and thus on the viability of the strain.

More elements of social interaction would also be interesting to add. They may be difficult to observe though, as the animals are likely to be disturbed by the investigator's presence or may hide in the nest. Testing more behavioural parameters by the animal technicians is outvoted by the time element. However, to increase the behavioural phenotyping of newly produced transgenic and mutant mice and to improve the animal welfare assessment, additional behavioural tests may be performed by the researcher. Several behavioural studies for the validity of the new mouse models have already been performed (Crawley and Paylor, 1997; Crawley, 1999; Rogers et al., 1999; Brown et al., 2000). In our previous studies on the implications of transgenesis for the well-being of mice, we have described some non-invasive and easy to determine behavioural tests, which were found to be discriminative for the detection of significant differences in behaviour (Van der Meer et al.,

1999, 2001b,c). These tests include, among others, pre-weaning behavioural tests, the hole board test (exploration and habituation), the cage emergence test (reaction to novel environment), the light-dark test (index of anxiety), circadian rhythm (by using the automated LABORAS device), climbing behaviour (adding a climbing object as cage enrichment) and response to handling (at and after handling in the home cage). Such battery of tests could be used for the careful monitoring of health and welfare of the transgenic offspring.

An alternative for the time-consuming weighing of the animals might be body condition scoring (BCS), which is a useful, rapid and practical tool for evaluating overall condition and health assessment of the mouse (Foltz and Ullman-Cullere, 1999). It can be scored during weekly cage cleaning, when the animals have to be handled anyway. BCS is particularly helpful in cases where pregnancy, organomegaly, or tumour growth may interfere with body weight assessment. In short, the mouse is picked up by its tail and the body condition is noted by passing a finger over the backbones, while, according to some observers, just looking at the animal will also give a quick indication. The body condition can be scored on a scale of 1-5; score 5 being obese without the ability to feel the back bones at all, while with score 2 until 1, the mouse is becoming thin, the bones are prominent and muscle wasting is advanced. A body condition score of 2 or 1 suggests a decline in overall condition and euthanasia is recommended. Considering BCS score and weight-loss may be useful. A weight-loss of 10-15% within a few days or an overall weight-loss of 20% are criteria for euthanasia (Foltz and Ullman-Cullere, 1999).

Finally, it is important to consider the inclusion of post-mortem parameters in the monitoring, such as general macroscopic inspection, weight of several organs (e.g. heart, kidney, liver, spleen) and further microscopic examination, when necessary, at the end of the experimental period, in order to evaluate the consequences of the introduction or knockout of genes for the well-being of the animals (Van der Meer et al., 2001c). The information gained by post mortem inspections can provide clues as to how the *in vivo* parameters should be interpreted as indicators for animal welfare.

Dealing with welfare problems

Whenever there are obvious indicators for welfare problems detected during the routine monitoring (e.g. behavioural, clinical or morphological), the animal welfare officer or veterinarian in charge should be notified. They are the persons to decide whether euthanasia or increased monitoring of the animal(s) is required. The score sheets form the basis for taking action with respect to welfare and will be a valuable tool for all persons working with the animals. When welfare is likely to be compromised for one or more animals, this can be easily visualised for other scorers and the researcher by using differently coloured labels on the cage, with the date of scoring on the back. The necessity of increased monitoring of the animal(s), for example on a daily basis, could also be implicated by using this system (W. Kort, personal communication).

The welfare assessment should be considered as an integral part of transgenic procedures and by including the recording of all abnormal observations in international databases and journals, it could contribute towards refinement in transgenic technology,

avoiding the use or wastage of extra animals. In this way subsequent users could be informed about adverse effects, how to recognise them, and which action to take.

Conclusion

During this study it has become clear that monitoring the welfare of transgenic mice using score sheets is both practical and useful. However, under the present circumstances in standard animal facilities, sufficient time in the animal technicians' daily schedule is the stumbling block. Therefore, the usefulness of the score sheets would not be increased by broadening the range of parameters. However, some parameters can be left out and/or exchanged by other parameters, depending on the specific characteristics of the mutant or strain. Subjective input can be reduced to a minimum by education of the scoring personnel.

The design of the score sheets needs to be flexible so they remain effective after the introduction of any changes. Animal technicians need to be instructed how to employ the scoring systems to the animals' best advantage and specific guidelines must be set-up to indicate how the results should be interpreted. Great care must be taken in instructing scorers as to the relevance of their results. Humane endpoints must be established i.e. the point when animal welfare has reached such a poor level that the experiment should end and in which cases the animals should be euthanised. In this way, monitoring the welfare of transgenic animals will provide a way of minimising animal distress. The score sheets described in this study can be used for routine monitoring, and can thus hopefully contribute to the design of the welfare assessment diary. They can also be very useful for training newer research staff and animal technicians in determining what to look for.

Recommendations

The following recommendations on the use of score sheets can be made based on this study:

- The practical value of the score sheets, as predictors of impending death as well as indicators of poor welfare, needs to be further confirmed in wider situations and subjected to retrospective analysis.
- Monitoring the welfare of transgenic mice using score sheets should be introduced on a routine basis.
- Animal technicians should perform the monitoring.
- Extra time should be made available in the animal technicians' timetables to include welfare monitoring.
- Screening should take place during both the pre- and post-weaning period.
- After weaning, the scoring can take place once a week during cage cleaning. Scoring parameters should be adjusted to the specific characteristics of the strain.
- Whenever obvious welfare problems are detected, the animal welfare officer or veterinarian in charge should be notified. Increased monitoring or euthanasia might be implicated.

- The number of parameters on the score sheets should be kept to the minimum needed for the purpose of the scoring.
- For most of the parameters, the number of possible answers should be limited to a maximum of three (+; -; and +/-).

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A. Score sheet day 0 - day 6

DNA construct: _____ Foster Mother: _____

Transgenic technique: _____ Expected effects: _____

Date of birth: _____ Litter size at birth: _____

Animal technician: _____

Day	0	1	2	3	4	5	6	Comments
Dead[1] date								
cannibalism								
Pups in nest[2]								
Pups out of nest[3]								
Milk spot[4]								
Abnormalities[5]								

[1] Number of dead pups. In case of cannibalism place a "+" in the box marked "cannibalism".
[2] Fill in the number of pups in the nest
[3] Fill in the number of pups out of the nest
[4] Fill in the number of pups that clearly have milk in their stomachs
[5] Any distinct traits or abnormalities can be recorded here

B. Score sheet day 10 / day 14

DNA construct: _____ Foster Mother: _____

Transgenic technique: _____ Date of birth: _____

Animal technician: _____ Date: _____

Individual mice	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Comments**
Identification mark[1]									
Dead[2]									
Cause of death									
In the nest[3]									
Weight (grams)									
Fur growth[4]									
Nipples[5]									
Upper incisors[6]									
Lower incisors[7]									
Walking[8]									
Righting[9]									
Ears open[10]									
Eyes open[11]									

[1] Fill in each animal's identification mark (for example: number, colour, ear clip, etc)
[2] To be completed if the animal is dead, if known, fill in the cause of death.
[3] + = the animal is in the nest, - = the animal is not in the nest.
[4] + = covered with soft fuzzy fur, - = little or no fur / bald patches.
[5] + = nipples are clearly visible, - = no nipples visible.
[6] + = upper incisors fully erupted, - = upper incisors not fully erupted.
[7] + = lower incisors fully erupted, - = lower incisors not fully erupted.
[8] + = mouse shows mature locomotion, stable and supported by all four limbs, - = any deviation.
[9] The animal is laid on its back. + = the animal immediately turns over onto all four limbs, - = any deviation.
[10] + = ears are open, - = ears are not yet open (only scored at day 14).
[11] + = eyes are open, - = eyes are not yet open (only scored at day 14).

C. Score sheet at weaning and after weaning

DNA construct: _____ Foster Mother: _____

Transgenic technique: _____ Date of birth: _____

Animal technician: _____ Date: _____

Individual mice	1	2	3	4	5	6	7	Comments
Dead[1] cause of death								
Identification mark[2]								
Posture[3] normal								
huddled up								
other abnormal position								
Reaction to cage opening[3] active (normal)								
hyperactive								
little/no response								
Reaction to handling[3] none (normal)								
irritation/biting								
fear (faeces/urine)								

[1]To be completed if the animal is dead, if known, fill in the cause of death.
[2]Fill in each animals' identification mark (for example: number, colour, ear clip).
[3]Select one of the three possibilities and mark it with a "+".

Continuation: C. Score sheet at weaning and after weaning

Date: _____

Individual mice	1	2	3	4	5	6	7	Comments
Weight (grams)								
Gender (♂/♀)[1]								
Fur[2] normal[3]								
pilo-erection								
bald								
other abnormalities								
Whisker chewing (+/-)[4]								
Abnormalities to eyes (+/-)[5]								
Walking [6] normal								
circles								
unable to stand on 4 legs								
other abnormalities								
Aggression after cleaning (+/-)[7]								
Unexpected phenotype								

[1] Fill in ♂ =male, ♀ =female.
[2] Select one of the four possibilities and mark it with a "+".
[3] Fur normal = smooth and shiny.
[4] + = confirmed / present, - = absent.
[5] + = abnormal eyes for example; red, closed, excessive tear production, - = no abnormalities.
[6] Select one of the four possible answers and mark it with a "+".
[7] + = animal is aggressive towards cage mates after cleaning, - = animal shows no aggression.

Chapter 7

General discussion

The production of transgenic animals, in particular of transgenic mice, has generated many new models for human diseases and has led to major breakthroughs in several biomedical research areas. However, due to uncertainties about the effects of biotechnological manipulations involved in the production of transgenic animals, and because changes in the genome are often unpredictable, concerns have been raised whether the welfare of animals produced by transgenesis is more at risk than of animals produced by changing the genotype through traditional breeding methods (Van Zutphen and Van der Meer, 1997).

During transgenesis, cells and embryos are subjected to various *in vitro* manipulations, including microinjection, electroporation, *in vitro* embryo culture and embryo transfer. These procedures can potentially cause welfare problems for the resulting offspring. To obtain more insight into this aspect, the (harmful) effects of the biotechnological procedures of transgenesis must be differentiated from the (harmful) effects of the expressed or mutated transgene. Therefore, in the present thesis, different groups of mice were generated, each of which underwent different aspects of either the microinjection technique (Chapters 2 and 3) or the gene targeting technique (Chapters 4 and 5), in order to determine whether the manipulations had any effects on the development or behaviour of the progeny. Specific effects of the different biotechnological procedures could be studied this way. In addition, the feasibility of the use of score sheets for monitoring the welfare of transgenic mice, as part of the daily care of the animals, has been explored (Chapter 6).

In the first part of this thesis, the question is addressed whether the various biotechnological procedures involved in the process of microinjection-induced transgenesis have an effect on the well-being of mice. Studies in sheep and cattle indicate that *in vitro* procedures as embryo culture, embryo transfer and nuclear transfer techniques, may cause an increase of birth weight, and several physiological and anatomical anomalies in the progeny (Wilson et al., 1995; Walker et al., 1996; Kruip and Den Daas, 1997; Van Reenen and Blokhuis, 1997; Cibelli et al., 1998; Renard et al., 1999; Hill et al., 1999; McCreath, 2000). Therefore, it could be expected that the various *in vitro* manipulations used for the production of transgenic mice would also affect health and welfare of these animals. The results revealed that the presence of the microinjected DNA-construct (both functional and non-functional) influences the survival rates of pups during the first 2-3 days after birth. In both groups, the average loss of pups was about 10%. In the groups without the DNA construct, none of the pups died. Although in our studies, the biotechnological procedures did not seem to influence the weight of the pups at birth, the increase in body weight during the first 11 days after birth was significantly higher for the non-transgenic pups after transgenic procedures, compared to the controls. They stayed heavier during the whole post-weaning period, but they did not show a higher growth rate. However, transgenic animals with a non-functional CRF construct did not differ in their body weight from the controls during both the pre- and post-weaning period. Major differences were found between the group of transgenic CRF mice and the other groups of animals. CRF mice showed differences in behaviour, weight gain, and morphology compared to control mice. These differences were all in line with the overexpression of the CRF gene, resulting in more anxious mice, that are less active and show features of Cushing's

syndrome, partly due to increased corticosterone levels (Boehme et al., 1997). No major pathology was found during post-mortem examinations for any of the treatment groups.

The results so far indicate that the biotechnological procedures of microinjection as such have no major effects on the normal development and thus on the welfare of the mice that survive the perinatal period. Although these results are reassuring, general conclusions should only be drawn when more and different transgenic lines have been studied in a comparable way.

In the second part of this thesis, the effects of the ES cell-meditated gene targeting procedures on behaviour and physiological/morphological development of the resulting offspring were studied. The results revealed that these procedures did affect some of the normal physiological and developmental processes of the progeny. An increased (perinatal) pup mortality was detected after the implantation of blastocysts, which were microinjected with ES cells (with or without the electroporation step). Furthermore, the birth weights of the offspring of these groups were higher than the controls, while during the rest of the pre- and post-weaning testing period, mice of all treatment groups were heavier than controls. The differences in post-weaning behaviour, observed for the two groups of chimeric mice (one derived from electroporated ES cells and the other from non-electroporated ES cells), when compared to the other (non-chimeric) groups, could be attributed to the genetic background of the 129/Ola strain from which the ES cells were derived, rather than to the biotechnological manipulations of the ES cells and/or blastocysts (Chapter 5). The high percentage of hermaphrodites (8%) and several other pathological lesions found among the chimeras, seems to indicate that developmental problems may occur when cells from different origin are simultaneously contributing to the development of one individual (Chapter 5). Therefore, it can be concluded that gene targeting procedures should always be accompanied by careful monitoring of health and welfare of the resulting offspring, including post mortem examinations of both chimeric and non-chimeric animals on a routine basis, in order to further evaluate the consequences of chimerism for the well-being of animals.

Besides the effects of the transgenic techniques on the welfare of animals, there are some aspects that also need to be considered in the production and use of transgenic and knockout mice. Firstly, the parental animals (superovulated females, vasectomised males, foster mothers) needed to produce the transgenic founders and chimerics, will also experience discomfort from the experimental procedures to which they are exposed, such as injections, anaesthesia, surgery and early mating (Chapter 1).

Furthermore, at weaning, the handling procedures for determining whether or not the offspring carry the transgene, can cause discomfort. Methods used for genotyping the mice include biopsy of ear or tail, or taking mucosa samples (which can be stressful to the animal, due to the restraint, and the forced opening and swabbing of the mouth). An alternative, less invasive method might be sampling of hair follicles (Schmitteckert et al., 1999). Each animal must be individually genotyped. This requires the labelling of the progeny. There are several methods for identification. Less favourable methods include toe clipping, ear tagging or

tattooing. Our method of choice, the use of colour marks, proved to be feasible and is not stressful for the animals.

As welfare can be compromised in the process of transgenesis, welfare assessment should be performed on all newly generated transgenic animals from birth onwards. Welfare indexes such as survival rate, morphological development, behavioural observations, life-span, growth rate, reproductive capability and post-mortem pathology can be useful not only to obtain insight into the possible impairment of well-being, but also to define the newly produced animal models and to refine the housing conditions and breeding protocols, in line with the animals' needs (Costa, 1997a,b). For the systematic observation of the impact of transgenesis on the well-being of the animals, a protocol was developed in the present study. Several of the parameters mentioned above were included in this protocol and various groups of animals, all with a different transgenic background, were screened. This approach was found adequate for monitoring a broad variety of behavioural and morphological characteristics. Most of the behavioural tests as employed in this study were sufficiently discriminative to differentiate between treatment groups and controls. Thus, these tests can also be used for behavioural phenotyping of other newly produced transgenic and knockout lines and to study possible effects on their welfare. The tests are simple, easy to perform and non-invasive. The exact order of testing does not appear to be critical. Individuals with some training in care and handling of rodents can easily learn to conduct the series of observations with accuracy and good inter-observer reliability. Equipment requirements are generally simple and inexpensive. Other, more comprehensive observational test batteries to evaluate the general health of new mouse mutants have been published elsewhere, however, these are much more laborious (e.g. Crawley and Paylor, 1997; Meyer, 1998; Rogers et al., 1999; Royle et al., 1999; Crawley, 1999, 2000; Bolivar et al., 2000; Brown et al., 2000). One has to keep in mind that in producing knockout mice, the differences of the genetic background of the parental strains can influence the behaviour of the (chimeric) animals (Gerlai, 1996), as has been explained in Chapter 5.

Monitoring the welfare of transgenic mice, as part of the daily routine of animal care in a transgenic unit, by using score sheets, has been found to be both practical and useful. The scoring system as described in this thesis (Chapter 6) contained a limited number of sensitive, easy to determine and non-invasive parameters, selected from our previous studies and may result in the early detection of animal welfare problems. It should, however, be kept in mind that the monitoring system, when performed by animal caretakers, is an additional task, on top of the regular workload. Extra time and funds should be made available if it is to be implemented successfully.

To reduce the need for extensive breeding, care and housing of large numbers of animals, it may be desirable to maintain one or more lines by cryopreservation (freezing) of embryos. This method saves time, money and space in the animal facility (Rall and Wood, 1994; Zhu et al., 1996). In addition, a scientific valuable line in which transgenic status has an adverse effect on animal welfare can be maintained for future research without causing continuos suffering. In principle, each mouse line can be preserved by cryopreservation, although survival rates may differ significantly between lines, including transgenic ones

(Ibáñez et al., 1997). Besides the preservation of lines, freezing of embryos can also be of interest for the distribution of transgenic mice. Stressful transport can be prevented and the chance of transmitting infectious diseases from one laboratory to the other is reduced. However, further studies into the side-effects of the different cryopreservation techniques and cryoprotectants is necessary, because the different techniques used for cryopreservation can have different welfare implications in later stages of life. Chromosomal abnormalities, metabolic changes and long-term effects on morphophysiological and behavioural features have been described (Van der Elst et al., 1995; Dulioust et al., 1995; Uechi et al., 1997). Also, the development of freezing sperm, containing the targeted gene mutation, can be an useful and cheaper approach for retaining and distributing valuable transgenic and knockout mouse lines (Crawley, 2000).

Microinjection technique

Pronuclear microinjection has by far been the most widely used transgenic method. Since its first application in animals in 1980, this technique has become well established, especially in mice, which are the most commonly used transgenic mammals. It is also currently the most practical and reliable method of producing transgenic livestock such as cattle, pigs, sheep, goats and rabbits. The technique of microinjection is reasonably efficient, foreign genes (from other species), if combined with the appropriate regulatory genes, are efficiently expressed. Moreover, there is no apparent limit to the size of the inserted DNA molecule.

There are, however, disadvantages associated with this technique (Pursel and Rexroad, 1993; Pinkert, 1997). Application of the technique requires highly skilled workers and involves expensive equipment. There is also no control over the site where the gene inserts itself into the genome of the recipient. This has several consequences. First, the integration site might influence the level of expression of the gene introduced. Moreover, the integration site will be different for each individual. Therefore, the transgenics can not mutually mate to obtain a homozygous transgenic line. Another disadvantage of microinjection is that the number of copies of the foreign gene fragment that become integrated is not known. Indeed, as many as 1,000 copies of the new gene may be found at a single chromosomal site. Under these circumstances it is not possible to study factors regulating gene expression, as it is not known which of the integrated genes is active. It is also important to realise that the time of integration may vary. If the integration does not take place before division of the zygote, only a proportion of the cells will carry the transgene and a mosaic will occur. The transgene is then present in part of the somatic cells and germ cells. Further, the integration may occur at the exon site of a gene, thus introducing an insertional mutation. The transgenic animal may then show differences in its phenotype, which is not directly caused by the transgene introduced. The frequency of these insertional mutations is not exactly known, because genetically deviant embryos often die at an early stage of gestation and are then rejected. Besides this, mutations are not always recognisable in the phenotype.

Gene targeting technique

The technique of embryonic stem (ES) cell-mediated gene transfer offers several advantages when compared to microinjection. The procedure of introducing the transgene takes place *in vitro* by homologous recombination and one can check the genome of the ES cells prior to their introduction into the embryo. Only those ES cells with the gene knocked out or integrated at the desired site, will be used. Methods exist to check whether the gene is actually present (marker genes). After inbreeding, it is possible to study specific gene functions with heterozygotes as well as homozygotes.

However, this technique also has disadvantages. It is the most intricate, costly and technically demanding method. To date, only functional ES cells have been derived from mouse embryos. Research is needed to apply this technique to species other than mice. The culture of ES cells *in vitro* is time consuming and labour intensive. A line of ES cells must be carefully monitored, to assure they retain normal chromosome number and arrangement, and guarantee that the cells have not become contaminated or differentiated. Because chimeras don't always contain the transgene in their germ cells, or only partly, selective and intensive breeding is often required to obtain the transgenics in appropriate numbers. Another disadvantage of the knockout approach is the lack of tissue specificity.

Future perspectives

The last 15 years emphasis has been on refining the technique by which the DNA is introduced into the mouse, and on increasing the predictability of the whole process. This has partially been achieved with the development of gene targeting techniques. Gene targeting in mice has already helped to determine the function of more than 800 genes (OECD, 1998). Many laboratories are now working on the production of ES cell lines or cell lines similar to ES cells from animal species other than the mouse. Also, cloning by nuclear transfer (Wilmut et al., 1997; Cibelli et al., 1998; McCreath et al., 2000) may be used to speed up the process of introducing transgenes into breeding livestock. However, so far, embryos resulting from nuclear transfer still have a high rate of mortality, and might be unusually large, so there may be welfare problems for both the offspring and the mother around the time of birth (Appleby, 1998). In addition, several researchers have reported developmental abnormalities associated with somatic cell nuclear transfer, e.g. high incidence of kidney defects, liver and brain pathology (Cibelli et al., 1998; Renard et al., 1999; Hill et al., 1999; McCreath, 2000). Although there are some indications that inappropriate expression of imprinted genes may be involved (Kono, 1998), detailed investigations are needed to further explore this phenomenon.

Techniques for conditional gene targeting have emerged, thus allowing the turning on or off of the gene of interest at certain stages of development, or in certain organs. An effective method for the creation of tissue-specific knockout mice is designed around the Cre/lox system (Müller, 1997; Rajewsky et al., 1998). The development of inducible knockouts enables research on mice that develop and mature under normal conditions until adulthood, with unaffected gene expression. Inducible mutations are turned on or are inactivated by crossing or by drug treatment. One strategy is the induction or inactivation of a tetracycline regulatory system, regulated by an antibiotic drug such as doxycycline (Blau and

Rossi, 1999). At a chosen time, mice are treated with tetracycline that binds to an inserted transactivator protein. This inactivates the transcription of the gene of interest. After a certain period the gene product will be absent, whereas no compensatory changes have taken place during development. A main advantage of this technique is the possibility to turn transcription of a particular gene on and off.

Current research is being performed to combine these two different techniques, which will lead to tissue-specific inducible knockout mice, in which the transcription and translation of a gene of interest can be turned on and off in predefined body tissues at a chosen time. This would potentially reduce animal suffering, as the resulting mouse mutants are completely normal until adulthood. As conditional/inducible knockouts become feasible, the targeted mutation technology is likely to become the major research tool for behavioural genetics, for many areas including neurobiology, and throughout biomedical research (Crawley, 2000).

A recent development is the production of chimeric animals by aggregation of ES cells with pre-implantation embryos. Kong et al. (2000) achieved aggregation of zona-free morulas (8-16 cell stage embryos) and ES cells, by co-culturing them overnight and subsequently transferring them into recipient animals. Their results demonstrate that aggregation of ES cells can be a convenient and cheaper alternative to microinjection when preparing chimeric animals. The efficiency of generating chimeric pups by morula aggregation (41%) was quite comparable to ES cell injection (38%) into blastocysts. In the future, the procedure may also be applicable to other species for which ES cell lines become available.

The production of transgenic animal models may eventually lead to a reduction in the use of (non-transgenic) laboratory animals. In the near future the use of transgenic animals is expected to increase. The net effect of transgenesis on the total use of laboratory animals is still unpredictable.

In the Netherlands, there has been a 53% increase (36,251 animals) in the use of transgenic animals in 1999, when compared to 1998. A total of 104,580 (14%) genetically modified animals were used in 1999 in animal experiments, mainly for fundamental research (Zo doende, 1999). Most of these animals were mice (95.2%), while only small numbers of rats (2.1%), rabbits (0.2%) and fish (2.4%) were used. In addition, for the production of the genetically modified mice and rats, 39,275 animals were used (including the donor mice, vasectomised males and foster mothers), which is an increase of 135% compared to 1998.

A large part of the present transgenic animal models is used for the study of the causes of disease processes in humans and to arrive at better opportunities for diagnosis, prognosis and therapy. There is a need for more fundamental knowledge. This is the main reason why the use of transgenic animals will probably still increase in the near future. For many diseases like cancer, heart and circulatory diseases, autoimmune diseases and several other disorders a relationship with DNA deviations has been demonstrated. Population-epidemiological research has also demonstrated that the occurrence of these diseases is often based on changes in hereditary material and a more direct approach to the genetic basis of these diseases is recommended.

Recently, a large part of the approximate 30,000 different human genes has been characterised and mapped (Venter et al., 2001; Ramser et al., 2001). The scientific challenge now is to find answers to the questions: What is the physiological function of all these genes, how do they co-operate and what happens in a biological sense at the level of the cell, organ or organism when mistakes in the genetic information occur? One way is to inactivate the equivalent genes in mice, or to substitute a mouse gene with a mutated version of the equivalent human gene. The abnormalities demonstrated by these mice should provide clues to the function of these genes. This type of research already has an important impact on biotechnology and is expected to contribute to an expansion in the use of transgenic animals in the near future.

Another reason why an increase in the total use of animals for transgenesis might be expected, is based on the fact that the feasibility and safety of diagnosis and therapy have to be thoroughly tested before they can be used in patients. For such research, there is often a need to generate animal models with a syndrome, which strongly resembles the human syndrome. The discomfort can, depending on the syndrome, be mild to severe. Therefore, it is recommended that the usefulness of the animal model is tested at a very early stage and that further emphasis is placed on the development and/or use of techniques which reduce discomfort by specific measures (e.g. minimising breeding; definition of humane endpoints, which allows early euthanasia; cryopreservation etc.).

Finally, although embryonic stem cell lines capable of producing germline chimeras are only available in the mouse at present, the technology of cloning by nuclear transfer will be used for the introduction of transgenes into breeding livestock and other species, like the rat. This might result in a greater variety of applications and a further increase in the use of animals.

Concluding remarks

The first and foremost strategy to avoid as much as possible the harmful consequences of transgenesis should be the systematic screening of all aspects of animal health and welfare; this provides the basis for critical decisions as, for example, the decision not to breed homozygotes, or to refrain from enhancing transgenic expression beyond a certain level. This welfare assessment should not only include the identification of the “intended” adverse effects (as for animal models of disease), but also the general monitoring of parameters that may be considered as indicators of the well-being of the animals, to discover non-intended side effects, as provoked by biotechnological procedures, by insertional mutations or interactions of genes. Several behavioural, physiological and morphological parameters have been presented in this thesis. Most of the (behavioural) tests as employed in this study can also be used for screening other newly produced transgenic and mutant strains, as to detect possible welfare problems. Serious attempts should be made not only to interpret the molecular/biological findings in terms of adequacy as animal model, but also in terms of potential risks for animal welfare, which urges the need for collaboration with welfare-orientated and/or behavioural scientists.

The score sheets as described in this thesis can be used for routine monitoring, and can thus, hopefully, contribute to the welfare assessment as required by the Dutch Inspectorate on Animal Experimentation. Hopefully, this kind of welfare assessment will reduce animal suffering by transgenesis in the future and lead to better refined animal models, and thus will be beneficial for both animals and researchers.

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Summary

Transgenic animals play an important role in biomedical research. Their use as animal model is still increasing. Although the process of transgenesis may contribute to refinement of animal use, the application of the biotechnological procedures that are involved in the production of transgenic animals may also cause unexpected, uncontrolled, and even undetected animal suffering. Therefore, the aim of the present thesis was to study the effects of the biotechnological procedures involved in genetic modification on the welfare of the resulting offspring. The two most frequently used techniques for inserting new genetic material into the mammalian germ line, pronuclear microinjection and embryonic stem (ES) cell-mediated gene transfer, have been studied. Different groups of mice were generated, each of which had undergone different aspects of the microinjection technique (Chapters 2 and 3) or of the gene targeting technique (Chapters 4 and 5), in order to determine whether the specific manipulations have any effect on the development or behaviour of the progeny. In this way, the effects of microinjection, electroporation, embryo-culture or embryo transplantation on the development of (chimeric) mice could be studied separately. All mice of the different groups were tested in their pre-weaning (0-3 weeks of age) and post-weaning period (4-30 weeks of age) for behavioural and morphological/physiological development and for clinical appearance. Thereafter, post-mortem examinations were performed. In addition, the feasibility of the use of score sheets for monitoring the welfare of transgenic mice, as part of the daily care of animals, has been explored (Chapter 6).

The first part of the study evaluated the effects of biotechnological procedures involved in the process of microinjection-induced transgenesis, by comparing four groups of C57BL/6 mice that differed in their transgenic background (transgenic after integration of a functional corticotropin-releasing factor (CRF) gene construct; transgenic after integration of a non-functional CRF gene construct (construct without promotor); non-transgenic after transgenic procedures; controls). The results of the pre-weaning test period (Chapter 2) revealed that the presence of the microinjected DNA-construct influenced the survival rate of pups during the first 2-3 days after birth. In both groups (one with a functional and one with a non-functional CRF gene construct), the average loss of pups was about 10%, while in the groups without the DNA construct, none of the pups died. Although the birth weights did not differ between groups, the increase in body weight of non-transgenic pups after transgenic procedures was significantly higher compared to the controls, but only during the first 11 postnatal days. These mice remained heavier during the whole post-weaning period. No significant differences in behavioural development were observed between the four groups of animals during the pre-weaning period. The CRF expression resulted in expected differences in morphological development between the transgenic mice and the other groups, like features of Cushings' syndrome (e.g. thinner hair and hair loss), due to increased corticosterone levels in these animals.

The same groups of animals were also monitored in their post-weaning period (Chapter 3). Major differences in behaviour, weight gain, and morphology were found between the transgenic CRF mice and the controls, in line with the overexpression of the CRF protein. CRF mice were more anxious and less active in various behavioural tests. For the

other treatment groups, no significant differences from control animals were found. No major pathology was found during post-mortem examinations for any of the treatment groups. The results so far indicate that, under the present conditions, the integration of a non-functional DNA construct, or the biotechnological procedures (microinjection; *in vitro* culture; embryo transfer) have no major effects on the development and behaviour of mice that survive the first 2-3 days after birth. However, before drawing general conclusions, more and different transgenic lines should be studied in a comparable way.

The second part of the study focused on the effects of the procedures involved in gene targeting on the behaviour and physiological/morphological development of the resulting (chimeric) mice. Six groups of mice (each undergoing specific aspects of the biotechnological procedure, including electroporation of embryonic (ES) cells, embryo culture, and/or microinjection of ES cells into blastocysts) and one control group, were compared. The control mice and all the blastocysts used were from the same inbred strain (C57BL/6), while the ES cells were derived from the 129/Ola strain.

As in the microinjection-induced transgenesis, the biotechnological procedures increased (perinatal) pup mortality and body weight. No further differences between the six treatment groups and untreated C57BL/6 controls could be attributed to the biotechnology procedures (Chapters 4 and 5). Therefore, we conclude that these procedures *per se* do not induce significant discomfort for the offspring that survive the first 2-3 days after birth. Chimeric mice were less explorative, more anxious and slower in their habituation than the other mice, but these are characteristics of the 129 strain and thus are not specifically caused by the biotechnological procedures. However, the occurrence of hermaphrodites (8%) and some other gross pathologies observed in both groups of chimeric animals, indicate that during the production of gene targeted mice, health and welfare of these animals should be carefully monitored.

Finally, a scoring system has been developed, containing a limited number of sensitive, easy to determine and non-invasive parameters, selected from our previous studies on implications of transgenesis for the well-being of mice (Chapter 6). The feasibility of this scoring system has been tested, to assess the use of score sheets for monitoring the welfare of transgenic mice on a practical basis, as part of the animal technicians' daily routine in a transgenic unit. It has been found to be both practical and useful. Therefore, in the production of transgenic animals, the use of score sheets is recommended, in order to detect both the intended and the non-intended (side) effects of the introduced or mutated gene at an early stage of development.

Samenvatting

Bij genetische modificatie door middel van transgenese worden met behulp van biotechnologische technieken erfelijke eigenschappen veranderd of van het ene naar het andere organisme overgebracht. Daarbij kan de natuurlijke barrière tussen soorten overschreden worden. Wanneer de nieuwe genetische informatie terechtkomt in de kiembaan en vervolgens van de ene op de andere generatie wordt doorgegeven, ontstaan transgene dieren. Er zijn verschillende methoden van transgenese.

De meest gebruikte methode is de micro-injectie techniek, waarbij een genconstruct met behulp van een micropipet in één van de twee voorkernen van een bevruchte eicel wordt geïnjecteerd. De techniek is redelijk efficiënt, maar de plaats van integratie in het genoom is willekeurig. Een andere veel toegepaste techniek is gene-targeting, waarbij gebruik gemaakt wordt van embryonale stamcellen (ES-cellen). Hierbij worden pluripotente ES-cellen uit een (pre-implantatie) embryo genetisch veranderd. Via homologe recombinatie kan een genconstruct uitgewisseld worden tegen een eigen gen. De plaats van integratie is daarmee bepaald. Deze cellen worden vervolgens weer ingebracht in de blastocoelholte van een (ander) embryo. Er kunnen hierbij ook zogenaamde “knock out” dieren worden gemaakt, waarbij via homologe recombinatie een gen naar keuze wordt uitgeschakeld. Hierdoor kan meer inzicht verkregen worden in de oorspronkelijke functie van het gen.

Transgene dieren spelen een belangrijke rol in het biomedisch onderzoek. De techniek schept de mogelijkheid om diermodellen te maken voor specifiek menselijke genetische ziekten. De meeste transgenese experimenten bij gewervelde dieren worden uitgevoerd met muizen. Het gebruik van de muis als transgeen diermodel neemt nog steeds toe. Door middel van transgenese kunnen zeer specifieke diermodellen gemaakt worden. Dit zou op den duur tot een vermindering en verfijning van het gebruik van (niet-transgene) proefdieren kunnen leiden. In de toekomst wordt echter een toename van het aantal transgene proefdieren verwacht, waardoor het uiteindelijke effect van transgenese op de totale omvang van het proefdiergebruik nog niet goed is te voorspellen.

Hoewel het proces van transgenese kan bijdragen aan de verfijning van het proefdiergebruik, kunnen de bij transgenese betrokken biotechnologische procedures ook leiden tot onverwachte, oncontroleerbare en zelfs niet direct zichtbare welzijnseffecten voor de dieren. Het doel van het in dit proefschrift beschreven onderzoek was dan ook om de effecten van de biotechnologische procedures op het welzijn van de nakomelingen nader te bestuderen. Verschillende groepen muizen zijn geproduceerd, die elk verschillende aspecten van ofwel de micro-injectie techniek (Hoofdstuk 2 en 3) ofwel de ES-cel techniek (Hoofdstuk 4 en 5) hebben ondergaan, teneinde vast te stellen of de specifieke manipulaties enig effect hebben op de fysiologische ontwikkeling of het gedrag van de nakomelingen. Op deze manier kunnen de effecten van micro-injectie, electroporatie, embryo-kweek of embryo-transplantatie op de ontwikkeling van (chimere) muizen elk afzonderlijk worden bestudeerd. Alle muizen van de verschillende groepen zijn getest in de periode vóór het spenen (0-3 weken oud) en een periode na het spenen (4-30 weken oud). Vervolgens is er post-mortem onderzoek uitgevoerd. Bovendien is het gebruik van scoringslijsten voor de bewaking van het welzijn van transgene muizen, als onderdeel van de dagelijkse verzorging van de dieren, geëvalueerd.

In het eerste deel van de studie zijn de effecten van de biotechnologische procedures welke een rol spelen bij het proces van micro-injectie geïnduceerde transgenese, bestudeerd. Vier verschillende groepen C57BL/6 muizen, die elk verschilden in hun transgenese achtergrond (transgeen na integratie van een functioneel corticotropin-releasing factor (CRF) genconstruct; transgeen na integratie van een niet-functioneel CRF gen construct (construct zonder promotor); niet-transgeen na transgenese procedures; controles) zijn met elkaar vergeleken. De resultaten van de test periode vóór het spenen (Hoofdstuk 2) toonden aan dat de overlevingskans van de pups gedurende de eerste 2-3 dagen na de geboorte door de aanwezigheid van het gemicro-injecteerde DNA-construct wordt beïnvloedt. In beide groepen (zowel met een functioneel als met een niet-functioneel CRF gen construct) was het gemiddelde verlies van pups ongeveer 10%, terwijl in de groepen zonder het DNA construct, geen van de nakomelingen stierf. Hoewel de geboortegewichten niet verschilden tussen de groepen, was de toename in lichaamsgewicht van de niet-transgene pups na transgenese procedures significant hoger dan de controles. Deze muizen bleven zwaarder gedurende de hele testperiode. Geen significante verschillen zijn waargenomen in gedragsontwikkeling tussen de vier groepen dieren gedurende de periode voor het spenen. De CRF expressie gaf de verwachte morfologische ontwikkeling van Cushing's syndroom (o.a. dunner haar en haarverlies) te zien, welke veroorzaakt wordt door toegenomen corticosteron gehaltes in deze dieren.

Dezelfde groepen dieren zijn ook bestudeerd in de periode na het spenen (Hoofdstuk 3). Belangrijke verschillen in gedrag, gewichtstoename en morfologie zijn gevonden tussen de transgene CRF muizen en de controle dieren. CRF muizen waren angstiger en minder actief in de verschillende gedragstesten. Bij de andere behandelingsgroepen zijn geen significante verschillen ten opzichte van de controles waargenomen. Bij post-mortem onderzoek zijn geen belangrijke afwijkingen gevonden. Uit de verkregen resultaten kan de voorlopige conclusie getrokken worden dat noch de integratie van een (niet-functioneel) DNA construct, noch de biotechnologische procedures (micro-injectie, *in vitro* kweek, embryo transfer) belangrijke effecten hebben op de ontwikkeling en het gedrag van muizen die de eerste 2-3 dagen na de geboorte overleven. Echter, voordat algemene conclusies kunnen worden getrokken, dienen meer transgene lijnen op een vergelijkbare manier bestudeerd te worden.

Het tweede deel van het proefschrift is gericht op het bestuderen van de effecten van gene targeting op het gedrag en de fysiologische en morfologische ontwikkeling van de resulterende (chimere) muizen. Verschillende groepen muizen, welke elk een specifiek aspect van de biotechnologische procedure hebben ondergaan (electroporatie van ES-cellen, embryo kweek en/of micro-injectie van ES cellen in blastocysten) en een controle groep zijn met elkaar vergeleken. De controle muizen en alle blastocysten die zijn gebruikt waren afkomstig van dezelfde inteeltstam (C57BL/6), terwijl de ES cellen afkomstig waren van de 129/Ola stam.

Net als bij de micro-injectie geïnduceerde transgenese werd gevonden dat de biotechnologische procedures de (perinatale) pup sterfte en het lichaamsgewicht verhoogden. Chimere muizen waren minder exploratief, angstiger en langzamer in hun habituatie dan de

andere muizen, maar dit zijn karakteristieken van de 129 stam, en zijn dus waarschijnlijk niet een gevolg van de biotechnologische procedures. Bij post-mortem onderzoek bleek dat 8% van de chimere dieren hermafrodiet was. Ook werden enkele andere pathologische verschijnselen geobserveerd. Geconcludeerd werd dat gedurende de productie van “gene targeted” muizen, ook de gezondheid en het welzijn van de chimere dieren zorgvuldig moet worden gemonitored.

Tot slot is een scoringssysteem ontwikkeld, gebaseerd op een beperkt aantal niet-invasieve parameters (Hoofdstuk 6). De toepasbaarheid van dit scoringssysteem is getest. Vastgesteld werd dat de scoringslijsten gebruikt kunnen worden voor de bewaking van het welzijn van transgene muizen, als onderdeel van de dagelijkse verzorgingsroutine.

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Curriculum Vitae

Miriam van der Meer werd op 24 april 1965 geboren te Soest. Aan de Dalton Scholengemeenschap te Den Haag behaalde zij in 1984 haar VWO diploma. Aansluitend studeerde zij Biologie aan de Universiteit Utrecht. Het doctoraalexamen werd behaald in 1990 en omvatte de hoofdvakken endocrinologie (Faculteit Biologie, Vakgroep Vergelijkende Endocrinologie) en immunologie (Academisch Ziekenhuis Utrecht, Vakgroep Klinische Immunologie). Na een korte werkperiode bij het Veterinair Microbiologisch Diagnostisch Centrum, werd in 1992 een onderzoek verricht naar de stand van zaken betreffende de ontwikkelingen van biotechnologische technieken ten behoeve van de aquacultuur, bij de Wetenschapswinkel Biologie aan de Universiteit Utrecht. Aansluitend was zij een aantal jaren werkzaam bij de Hoofdafdeling Proefdierkunde als toegevoegd onderzoeker, eveneens aan de Universiteit Utrecht, alwaar zij literatuurstudies en interviews verrichtte betreffende de inventarisatie van technieken en toepassingen en gezondheids- en welzijnsaspecten van transgene dieren, geïnitieerd door de Nederlandse Vereniging tot Bescherming van Dieren en de ministeries van LNV en VWS te Den Haag en de Europese Commissie (Directorate General XII) te Brussel. In 1996 werd in samenwerking met Solvay Pharmaceuticals te Weesp een promotie project gestart bij dezelfde Hoofdafdeling Proefdierkunde, met als opdracht het bewerken van de in dit proefschrift beschreven vraagstelling.