Refinement of Mouse Husbandry for Improved Animal Welfare and Research quality

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Abstract

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Following advances in genetic engineering, mice are today the most commonly used mammal in research. This thesis addresses two issues: the impact on animal welfare and research quality by environmental enrichment and the characterization of genetically modified mice. In the first paper, the effects of environmental enrichment before weaning were studied. No alterations were found at age 4 weeks, but after 8 weeks of environmental enrichment the home cage activity had decreased. In the second paper, a battery of behavioural tests was used to characterize a spontaneous mouse mutant, the *leaner* heterozygous. This mutant showed cognitive and motor impairment. This finding was followed up, in the third paper, by housing the *leaner* heterozygous and wild type mice in cages with three different degrees of environmental complexity. In Morris watermaze tests at 6, 12 and 20 months of age, environmental enrichment was found to increase learning and memory capacity in mice of both genotypes.
In paper IV, a characterization routine is presented for pre-weaning monitoring of mutant mice. Individual pups were scored for several development parameters on postnatal days 1, 3, 7 and 14. At weaning (day 19 to 21) a clinical examination and the novel cage test for emotional reactivity were carried out. The protocol was tested in several C57BL/6, BALB/C, 129S6 and B6CBAF1 litters. The characterization routine was efficient in detecting differences between genotypes in weight gain, physical condition at weaning, reaction to handling and emotional reactivity.
In paper V, C57BL/6, 129S6 and B6CBAF1 mice were used. The mice were tested in the novel cage (at weaning), the open Field test (at 5 weeks), and from 9 weeks of age in the elevated plus-maze, the concentric square field and the rat exposure test. Results obtained with the novel cage test at weaning were largely consistent with those obtained in the established tests later in life.
In conclusion, this thesis shows that cage enrichment decreases the cognitive deficits in the heterozygous *leaner* mouse. The new protocol for pre-weaning monitoring can be used to detect deviations in early development and emotional reactivity in a number of commonly used mice genotypes.

Keywords: Refinement, husbandry, phenotyping, environmental enrichment, welfare, mice, emotional reactivity

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Contents

Appendix

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Marques, JM and Olsson, IAS. 2007. The effect of preweaning and postweaning environment on the behaviour of the laboratory mouse (*Mus musculus*). *Laboratory Animals* 41(1): 92 – 102.

II. Alonso, I, Marques, JM, Sousa, N, Sequeiros, J, Olsson, IAS and Silveira, I. Motor and cognitive impairments in the leaner heterozygous, a Cav2.1 voltage-gated Ca2+ mutant. Neurobiology of Aging (2007), doi: 10.1016/j.neurobiolaging.2007.04.005. *In Press*.

III. Marques, JM, Alonso, I, Silveira, I, Santos, C and Olsson IAS. The effect of environmental enrichment on the cognitive impairment of the leaner heterozygous. *Submitted*.

IV. Marques, JM, Ögren, SO, Augustsson, H and Dahlborn, K. A characterization routine for improved animal welfare in pre-weaning mice. *Manuscript*

V. Marques, JM, Olsson, IAS, Ögren SO and Dahlborn, K. Evaluation of exploration and risk assessment in pre-weaning mice using the novel cage test. Physiology & Behavior (2007), doi: 10.1016/j.physbeh.2007.08.006. *In Press*.

Introduction

The 3 R's –Replacement, Reduction Refinement, were introduced by William Russell and Rex Burch in 1959. The 3 R's have influenced legislation concerning laboratory animals and constituted the guidelines for research with laboratory animals. Russell and Burch defined Replacement as '*any scientific method employing non-sentient material which may in the history of animal experimentation replace methods which use conscious living vertebrates*', Reduction as '*means of lowering the number of animals used to obtain information of a given amount and precision*' and Refinement as '*any development leading to a decrease in severity of inhuman procedures applied to those animals which have to be used*'. Later, Smyth (1978) formulated the 3 R's definition of alternatives as '*all procedures which can completely replace the need for animal experiments, reduce the numbers of animals required, or diminish the amount of pain or distress suffered by animals in meeting the essential needs of man and other animals*'.

The laboratory mouse (*Mus musculus*) is the most commonly used vertebrate in research. While several characteristics of this species account for this preference – short life span, rapid development, short reproductive cycles and large litters, small body size and possibility for group housing – the main reason to why the use of mice has become so popular and increased in the last decades is the possibility and relative ease to modify its genome (Hudson, 2007). The development of techniques for gene manipulation has made it possible to create a large and increasing number of genetically modified mice, which are used for functional studies of specific genes and as models of human disorders. For this purpose, the number of mice used in research is likely to further increase in the next decade.
This thesis investigated the impact of two major issues within refinement - housing environment and phenotypic characterization of mutant mice - on the improvement of animal welfare and research quality.

Animal welfare

The term animal welfare is used to express the ethical concerns regarding the use and treatment of captive animals rather than as a scientific based concept (Duncan & Fraser, 1997). Several attempts have been made to study and define animal welfare in a scientific way. The functional based approach defines animal welfare in terms of the normal or satisfactory biological function of the animal, and relevant measures include health, reproductive success, occurrence of stereotypies, longevity and biological fitness. Within this approach, Fraser & Broom (1990) defined animal welfare in terms of the animal's capacity to compensate for environmental challenges through physiological and behavioural adjustments - homeostasis. In a complex environment the animal '*may fail to cope in that its fitness is reduced and either it dies, or it fails to grow or its ability to reproduce is reduced in some direct way. The welfare of an animal is its state as regards its attempts to cope with its environment*'.

An emotional based approach defines animal welfare in terms of subjective experiences. Here relevant research methods include measures of animals' preferences or motivations as well as physiological indicators of emotional states. One example is the definition proposed by Duncan (1993), who stated that '*neither health nor lack of stress nor fitness is necessary and/or sufficient to conclude that an animal has good welfare. Welfare is dependent on what animals feel*'.

A third approach to define animal welfare focuses on the nature of the animals and emphasizes that in order to promote welfare, animals should be provided with possibilities to express their natural behavioural repertoire. Relevant measures include the study of the animal's behaviour in the wild and the comparison of their behavioural repertoire to that of their captive counterparts. Rollin (1993) proposed that each animal species has an inherent genetically encoded 'nature', which he called 'telos' (e.g. it is in the nature of birds to fly), therefore, in order to promote welfare, we should raise animals in ways that respects their natures. He stated: '*Not only will welfare mean control of pain and suffering, it will also entail nurturing and fulfilment of the animal's natures, which I call telos*'.

Recently a new proposal by Korte et al. (2007) highlights the need to develop a new, more scientific definition of animal welfare where the concept of animal welfare is based on allostasis – stability through change, as opposed to the earlier presented homeostatic approach (Fraser & Broom, 1990). Rather than to maintain a constant internal state allostasis involves mechanisms that change the controlled physiological variable by predicting what level will be needed to meet an anticipated demand. '*Not constancy of freedoms but capacity to change is crucial to good health and good animal welfare*' (Korte et al., 2007).

Regardless of the different approaches to define animal welfare, it is generally agreed that if animals are subjected to unnecessary suffering during an experiment, the compromised welfare will interfere with the experimental results (Morton & Hau, 2002). Good animal-based research should use normal, healthy subjects, unless the illness in itself is the subject of investigation (Poole, 1997). It is, therefore, important to distinguish between the unavoidable pain and distress which are consequences of the research, and that which can be minimized by application of better experimental techniques and refined husbandry, reducing the variability of results and consequently, the number of animals required.

Research quality

Measured parameters must be valid, reliable and replicable to be of high scientific quality (Garner, 2005). Research quality concerns the ways to grant experimental outcomes with *validity* – the extent to which a measurement actually measure what the researcher wishes to measure, *reliability* – the extent to which a measurement is repeatable and consistent, and *replicability* – the extent to which a result can be repeated independently in different laboratories (Martin & Bateson, 1993). While reliability and replicability are mostly dependent on the experimental techniques used, the validity of results depends on the animal model and is likely to be affected by suffering, distress and abnormal behaviour (Poole, 1997, Garner, 2005, Würbel, 2001, 2007).Validity may therefore be enhanced by means of

refinement techniques which help to minimize the confounding effects of pain, distress and abnormal behaviour on experimental outcomes.

Refinement

Given that certain types of research can presently not be replaced by non-animal models, refinement studies are important to ensure an ethically acceptable use of animals: causing minimum animal suffering and distress and producing the best experimental results. Refinement includes the implementation of measures that avoid unnecessary suffering (e.g. recommendation of humane endpoints) and the improvement of experimental techniques (e.g. the use of anaesthesia and analgesia for animals which undergo painful experiments).
The work in this thesis focuses on two issues within refinement which have been found to have a major impact on both animal welfare and research quality - the implementation of environmental enrichment and an early characterization routine for improving animal welfare in genetically modified mice.

Mouse husbandry and housing

Husbandry concerns the way in which mice are kept, not only regarding their housing environment (e.g. light conditions, temperature, cage size, group size), but also the routine practices that they are subjected to (e.g. health monitoring, cage cleaning). While a scientific experiment that a mouse is subjected to may only last for a short time, laboratory mice spend most of their life in their home cage. Husbandry routines, therefore, play a fundamental role in both mouse welfare and the quality of the experimental results. The common husbandry routines for laboratory mice include housing in standard cages and ensure the good health and physical conditions of the animals, except in cases where an induced disease is part of the research. However, there is room for improvement regarding housing environment to minimize discomfort and to provide ways for the animals to perform species-specific behaviours. Furthermore, in this context it is important to implement monitoring routines of the animals. The detection of cases where mice suffer from discomfort, fear or distress may help to adapt improved husbandry routines to specific needs (e.g. provision of nutritive supplements to mice that do not feed properly), which will increase the quality of the results obtained.

Environmental enrichment

Environmental enrichment has been widely used in behavioural neuroscience since Hebb's proposal that brain functions and neural activity can change by experience (1947), and that the environment influences the development of the brain (Rosenzweig et al., 1978). Supplementing the cage with different items was found to have an impact on brain structure and morphology, by increasing perikaryonal and nuclear sizes in the cortex (Diamond et al., 1967) and increasing brain weight and size (Rosenzweig & Bennet, 1969). Furthermore, enrichment enhances neural development in the dentate gyrus, increased gliogenesis, synaptogenesis and angiogenesis (Van Praag et al., 2000). Later studies reported

that increased complexity also has an important role in delaying the onset and progression of several neurodegenerative diseases such as Huntington's (Hockly et al., 2002), Alzheimer's (Arendash, 2004), Parkinson's (Faherty et al., 2005), and Fragile X syndrome (Restivo et al., 2005) in animal models.

Environmental enrichment has been used since the 70s for zoo animals (e.g. Erwin et al., 1976, cited by Newberry, 1995). However, it was not until the early 90s that the provision of a more species- specific home cage environment became a common practice for improving the welfare of laboratory rodents (Würbel, 2007). Since then, it has been widely used, and a review written by Olsson & Dahlborn (2002) presents the wide variety of strategies used for mice in different laboratories.

Because of its distinct applications in neurobiology and animal welfare, the term environmental enrichment has been used inconsistently in scientific literature. In studies of brain plasticity, enrichment is defined as any '*combination of complex inanimate and social stimulation*' (Rosenzweig et al, 1978), whereas from a welfare point of view enrichment is '*the improvement of biological functioning of captive animals resulting from modifications to their environment*' (Newberry, 1995). The relevant measures of biological functioning proposed by Newberry (1995) include animal health, lifetime reproductive success and fitness. To serve the purposes of refinement by actually improving animal welfare, environmental enrichment must have a functional value for the species under consideration. The purpose is to provide the home cage environment with crucial features, so that natural behaviours can be expressed and reinforced (Blanchard & Blanchard, 2003, Baumans, 2005). It is also important to allow the animals to exert some control over their environment (Olsson & Dahlborn, 2002), which in turn promotes their ability to cope with novelty and scientific experiments (Young, 2003). With this in mind, Würbel (2007) suggested a classification for enrichment strategies where *pseudo-enrichments* do not have a biological relevance, do not improve welfare and may actually impair it if the objects provided constitute a source of stress (e.g. introduction of marbles or ping-pong balls). In contrast, *conditionally beneficial enrichments* are biologically relevant but the effects on animal welfare may differ with strain, sex or management (e.g. provision of a nestbox), while *beneficial enrichments*, are both biologically relevant and provide a real enhancement of welfare (e.g. nesting material).

Different types of environmental enrichment may also have a different impact on research quality in terms of data validity. Several authors (Würbel, 2001, Garner, 2005) argue that standard housing results in abnormal behaviour such as stereotypies, which are associated with an abnormal physiology. Thus animals reared under enriched environments make better research models. However, others have found enrichment to result in an increase in the variation of several physiological parameters measured experimentally, like body and organ weights (Eskola et al., 1999, Mering et al., 2001, Tsai et al., 2006). Results are contradictory, as several other authors have found that enrichment does not affect experimental mean values or inter-individual variation (van de Weerd et al., 2002, Augustsson et al., 2003, Marashi et al., 2004, Wolfer et al., 2004). This suggests, once again, that the impact on the scientific outcome is dependent on the type of enrichment, duration of the experiment and on the sex and strain of the animals (Tsai et al., 2002).

Garner (2005) argues that the opposition to the beneficial effects of environmental enrichment lies in the assumption that animals housed under standardized conditions are more normal than enriched animals. If this assumption is proven wrong environmental enrichment may help to address the potential effects of abnormal behaviour and physiology on scientific experiments thus improving the quality of experimental results.
Further studies are needed to understand the impact of different types of enrichment on animal welfare (Benefiel et al., 2005), but it is generally agreed that when applied correctly, enrichment has beneficial effects in reducing anxiety-like behaviours and stress responses (see Fox, 2006 for a review). This meets the conditions for both Refinement and Reduction (Van de Weerd et al., 2004). The review conducted by Olsson & Dahlborn (2002) draws attention to the fact that the answer for a correct use of enrichment lies on the selection of enrichment strategies that have a biological meaning to each species and exert no detrimental effects on the variability and validity of the scientific outcomes.

Mouse phenotyping and welfare monitoring

The use of genetically modified mice represents an important tool for advances in biomedical research. With the progresses in genetic engineering, it is now possible to produce an increasing number of mouse lines with genetic modifications obtained through transgenesis, or gene targeting techniques (knock-in, knock-out technology). Transgenic techniques allow the insertion into the genome of new or extra copies of genes, which may be derived from a different species. Two techniques are frequently used for the production of genetically modified mice – the microinjection and the genetic manipulation of embryonic stem cells. With microinjection, an extra gene is added to the genome in order to study the expression of new genetic material (van der Meer, 2001). With this method, the transgene is inserted in a random site, which may happen to be a functional sequence, where it will disrupt a gene in the original genetic makeup causing an *insertional mutation.* On the other hand, embryonic stem cells are used for site directed mutagenesis by homologous recombination (Overbeek, 1994). This process involves a modification of an existing gene with the transgene in a precise and predetermined way (one example is the production of a 'knockout', where the existing gene is switched off).
These advances have resulted in an accumulated need to develop ways to characterize the phenotype of the genetically modified mice, both from a scientific point of view (to serve the purpose of the scientific experiment) and for monitoring the possible occurrence of welfare problems which can be results of an unexpected phenotype.
A phenotype is the manifestation of a genotype, the mouse's genetic composition, in the context of environmental or epigenetic influences affecting gene expression (Linder, 2006). The phenotypes of genetically modified animals may partly result from side effects of the genetic manipulation (Mertens & Rülicke, 2000) or from other genetic or environmental factors interacting with or influencing the genetic modification (Barbaric et al., 2007). The genetic background, for example, is known to greatly influence the phenotypic expression (Gingrich & Hen, 2000,

Gerlai, 2001, Schalkwyk et al., 2007). The phenotype results from interactions with the background genes and other unknown mutations in the background phenotype (Crawley et al., 1997). On the other hand, several authors have drawn attention to possible compensatory processes (up- or downregulation of gene products) which may provoke secondary phenotypic changes, in particular, in the case of gene targeting (Gerlai, 1996, Gingrich & Hen, 2000, Gerlai, 2001). Besides the techniques presented above, cross breeding of different mutant lines is becoming increasingly common (e.g. Van Damme et al., 2005, Ip et al., 2006, Meng et al., 2007). This technique is likely to increase the possibility of unexpected phenotypes, since several genes will be involved in the process, which will cause a decrease in the occurrence and efficiency of compensatory mechanisms.

Several studies performed by van der Meer and colleagues (van der Meer et al., 1999, 2001a) have looked at the effect of gene manipulation techniques on the early development, behaviour and welfare of mice. Overall, they found no effects on mice welfare or pre-weaning development caused by the techniques used. A study performed by Thon et al (2002), analysing the reports to the Danish Animal Experiments Inspectorate reported that 36% of the used genetically modified strains had welfare problems, where 21% were affected to a minor degree, and 15% to a severe degree. 34% of the cases were reported as needing special care (e.g. special housing, special diet or rejection of the animal model), and the most frequently reported action was euthanasia. While some of the welfare impairments reported were expected as part of the genetic manipulation, other were unexpected side effects (e.g. a cancer model was unable to feed properly due to malformations of the skull). Another screening program conducted in Italy, showed that 5 out of 10 newly generated genetically modified mice had developmental defects (Costa, 1995, cited by Thon et al., 2002), and a surveillance program conducted at the Washington University identified both transgenic and knockout lines with unexpected tumour incidence, diabetes, hydrocephalus, epilepsy, osteoporosis, among others (Van Hoosier, 1999, cited by Thon et al., 2002).

Several test batteries have been developed as a means to extensively characterize genetically modified mice, such as the SHIRPA (Hatcher et al., 2001), and the EMPReSS (http://empress.har.mrc.ac.uk/). These batteries include tests for sensory, motor and cognitive functions and are usually applied during adult age. However, the main focus of these batteries is the characterization of phenotypical characteristics that are relevant for the research model. Other aspects of the phenotype that have an impact on animal welfare but not on the subject of interest are mostly overlooked (Brown & Murray, 2006).

Mouse pups are born poorly developed, and during the pre-weaning period there is a rapid physical and sensory development, with most sensory and behavioural characteristics maturing before postnatal day 15 (Fox, 1965). Most neurochemical transmitter substances and their receptors involved in brain emotional systems are fully developed at weaning. Therefore, it has been suggested that screening of mouse mutants during the pre-weaning period may be of crucial importance for the detection of developmental abnormalities that cannot be observed during adult life (Branchi & Ricceri, 2002). Several authors (van der Meer & van Zutphen, 1997, Mertens & Rülicke, 1999, 2000, van der Meer et al., 2001b, Dennis, 2002) have pointed out the need to develop proper protocols for routine welfare

assessment to be applied during the pre-weaning period. Such methods would provide a means to refine the animal model through the recommendation for improved husbandry or relevant humane end-points (Jegstrup et al., 2003).

Emotional reactivity

The concern for animal welfare is often based on the assumption that animals are capable of experiencing sensations and emotions (Mendl & Paul, 2004). Emotions, in both humans and animals are very diverse and involve many bodily and mental processes. At present there is no general accepted agreement on the definition of the term 'emotion'. In everyday language we use the term to refer to human moods and feelings and the way these are expressed both in overt behaviour and on the body level (Kandel et al. 1995). Therefore, emotions can be defined as (transient) mental states which manifest themselves in a number of physiological and psychological (behavioural) processes. In animals too, emotions like 'fear' or 'anxiety', are regarded as comprising behavioural, physiological, cognitive and subjective components (Lerner & Keltner, 2000). Thus measures of animal emotions focus primarily on the physiological and behavioural components of the emotional response (Mendl & Paul, 2004), i.e. the physical and behavioural expression of what may resemble a human emotion. This is the origin of the term 'emotional reactivity'. Emotional reactivity is sometimes referred to as 'emotionality','anxiety' or 'anxiety related behaviour' and is widely used in pharmacological research. Emotional reactivity is assessed in a variety of exploration-based tests based on the idea that an animal that readily explores a novel environment is a 'non-emotive' one (Roy & Chapillon, 2004). Exploration has mainly been measured by different kinds of locomotor behaviours.This has later been complemented by the introduction of risk assessment/risk taking and approach/avoidance behaviours as valid measures of emotional reactivity (Rodgers & Dalvi, 1997, Rodgers et al., 1999, Augustsson et al., 2005).

Aims of the thesis

The overall aims of this thesis were to increase our knowledge about existing applications of environmental enrichment and phenotyping, and to develop a new routine for monitoring genetically modified mice and spontaneous mutants during the pre-weaning period, with special consideration for animal welfare.
Specific aims of the included papers were:

Paper I

- To investigate the effect of environmental enrichment during the pre-weaning and post-weaning period on mouse behaviour in adulthood

Paper II

- To assess motor and cognitive functions in the *leaner* heterozygous mouse throughout aging

Paper III

- To investigate the effect of environmental enrichment on the cognitive impairment of the *leaner* heterozygous mouse throughout aging

Paper IV

- To develop a score-sheet for pre-weaning characterization of mouse mutants
- To test the efficacy of the developed score-sheet in detecting welfare problems/differences in development in strains/genotypes commonly used as backgrounds from genetically modified mice (129S6, BALB/C, C57BL/6 and B6CBAF1)

Paper V

- To test the efficiency of the novel cage test in the evaluation of emotional reactivity in mice at weaning, by comparing its results with those obtained during adulthood.
- To compare the results obtained with the novel cage test with the outcome of several different tests for emotional reactivity.
- To evaluate the efficiency of the novel cage test in detecting differences in emotional reactivity in different strains/genotypes of mice commonly used as backgrounds for genetically modified mice (129S6, C57BL/6 and B6CBAF1)

Theoretical considerations to the aims of the thesis

Housing environment at an early age

While a considerable amount of work has been conducted on the effects of environmental enrichment on behaviour (e.g. Garner & Mason, 2002) and physiology (e.g. Chapillon et al., 2002) in adult mice, little is known about how housing environment during early life affects the behaviour of mice both as young and adults.
Studies in rats have shown that when exposed to a more complex environment during the pre-weaning period, adult rats perform better in problem-solving tasks (Ivinskis & Homewood, 1980) and show decreased stress responses (Parfitt et al., 2004), but there are no comparable studies for mice. Paper I addresses the use of environmental enrichment in mice during the pre-weaning period, and its potential effects on behaviour in adult life.

Characterization of the leaner heterozygous mouse

The spontaneous mutant *leaner*, which has a mutation in the $Ca_v2.1$ voltage-gated calcium channel, has been described for the first time by Sidman and colleagues (1965). Homozygous animals present severe ataxia due to cerebellar atrophy resulting from a gradual degeneration of granule, Purkinje and Golgi cells (Herrup and Wilczynski, 1982, Frank et al., 2003, Lau et al., 2004). Heterozygous animals do not present evidence for phenotypic abnormalities and have been described as normal (Fletcher et al., 1996) However, the diversity of symptoms in humans carrying the same CACNA1A mutation (Alonso et al., 2003) and the role of calcium homeostasis in normal brain function, indicated the possibility that subtle phenotypic alterations might exist in heterozygous mice. In Paper II, a systematic battery of tests was used to study the *leaner* heterozygous mouse, which included tests for motor and cognitive performance commonly used in phenotyping protocols.

Effects of housing environment on learning and memory in the leaner heterozygous mouse

Increased home cage complexity has been found to alter the brain structure and improve cognitive performance (van Praag et al., 2000). It has therefore been widely used in studies of brain plasticity (e.g Kempermann et al., 2002). In fact, complex environments have been shown to have beneficial effects on the onset and progression of many neurodegenerative disorders modelled in animals, such as Huntington (Spires et al., 2004), Alzheimer and Parkinson (Li & Tang, 2005) and Fragile X Syndrome (Restivo et al., 2005). These findings are the rationale behind the hypothesis that environmental enrichment might have an impact on the onset and progression on the cognitive impairment of the *leaner* heterozygous mouse. In Paper III we studied the effect of three types of housing, with different

space availability and physical complexity, on the cognitive performance of the *leaner* heterozygous at different ages.

Score-sheets for early phenotyping

Phenotypic characterization at an early age is important to detect gene effects that may not be detectable during adult age, and to identify possible compensatory or unexpected effects of the genetic manipulation (Branchi & Ricceri, 2002). However, most protocols for characterization of genetically modified mice, such as the SHIRPA or the EMPReSS are meant to be applied in adult animals (e.g. Rodgers et al., 1997), and focus on measurements of scientific interest for the specific animal model. Some authors have previously addressed the need to implement routines for monitoring welfare aspects, and have presented suggestions for score sheets (Mertens & Rülicke, 1999, van der Meer et al., 2001b). However, at present none of those procedures has been validated and either they are too complex to apply routinely, or they lack relevant information needed to establish actions to be taken such as humane endpoints (Jegstrup et al., 2003). In Paper IV we developed and tested a protocol for early characterization of induced and spontaneous mutant mice, to be used at production and breeding facilities, as part of the normal husbandry procedures.

Phenotyping emotional reactivity

Emotional reactivity involves both physiological and behavioural responses, and is therefore evaluated in exploration-based paradigms. Altered emotional reactivity has been shown to be associated with some lines of genetically modified mice (e.g. Sakić et al., 1994). It is therefore important to consider when developing protocols for characterization of genetically modified mice. Tests for emotional reactivity are included in the SHIRPA and EMPReSS protocols, but the complexity of the test arenas may not be suitable for routine testing in young animals. In Paper V the novel cage test was introduced as a routine to measure emotional reactivity in mice at weaning.

Materials and methods

Animals

Wild type mice

Three inbred strains of mice (C57BL/6, BALB/C and 129S6) and one hybrid (B6CBAF1) were used for the work in this thesis. The choice of strains for papers IV and V was based on the fact that those are often selected as background strains for genetically modified animals as well as in known strain differences in emotional reactivity which allowed for a 'genetic' validation, through strain comparisons, of the novel methodologies proposed in this thesis.

C57BL/6

This is the most widely used strains in research, both as a general purpose strain, and for the generation of congenics carrying both spontaneous and induced mutations (http://jaxmice.jax.org/info/index.html). The good performance of this strain in learning and memory tests (Holmes et al., 2002, Brooks et al., 2005), makes it a background of choice for models studying cognitive function and its highly exploratory profile allied to low emotional reactivity (Lepicard et al., 2000, Rodgers et al., 2002), which underlies its wide use for studies on anxiety-like behaviour. In Papers I – III, C57BL/6J were acquired from Harlan Iberica, whereas in Papers IV – V C57BL/6Bkl mice were acquired from Scanbur-BK.

129S6

Several 129S6 substrains are typically used to produce embryonic stem cells for gene targeting (Homanics et al., 1999, Võikar et al., 2001, Rodgers et al., 2002). The 129S6 strain has a substantial genetic and phenotypic variation, with many substrains available.

In general, 129S6 mice present a contrasting behaviour profile when compared to C57BL/6. It shows low exploratory behaviour and high emotional reactivity (Võikar et al., 2001, Holmes et al., 2002, Rodgers et al., 2002). In exploration-based tests of emotional reactivity, 129S6 mice are less active and generally more 'anxious' than their C57BL/6 counterparts (Crabbe et al., 1999, Rogers et al., 1999, Mcllwain et al., 2001) and there is a general opinion that this strain perform poorly in learning tasks. However, some substrains like the 129S6 have been found to have a good performance in several learning and memory tests (Holmes, 2002). In Papers IV and V the 129S6 was one of the selected strains for the genetic validation of the score sheet for early phenotyping and the novel cage test.

BALB/C

This strain is known to have a high emotional reactivity (Belzung et al., 2000, Lepicard et al., 2000, Ohl et al., 2001). Previous studies, performed by Belzung & Griebel (2001), indicated that BALB/C mice are good models for trait anxiety since it was the only strain that consistently showed higher levels of emotional reactivity when compared to other strains.

B6CBAF1

The use of F1 hybrids is becoming increasingly popular, and some of their characteristics account for this choice - F1 hybrids are genetically and phenotypically uniform, and they possess hybrid vigour, which makes them more resistant to disease, survive better under stress, to live longer and have larger litters than either of the parental strains (http://jaxmice.jax.org/info/index.html).

Spontaneous mutants

A spontaneous mutant was used in Papers II and III - the *leaner* heterozygous mouse ($tg^{la}/+$). *Leaner* mice have a spontaneous mutation in the $Ca_V2.1$ voltage-gated calcium channel which in humans is known to be associated with three dominantly inherited disorders: spinocerebellar ataxia type 6, familial hemiplegic migraine and episodic ataxia type 2 (Ophoff et al., 1996, Zhuchenko et al., 1997).

Behavioural tests

Several behaviour tests were used in this work.

- Tests for sensory development (Paper IV): righting reflex, nest finding, grip strength, vertical pole, position reflex, visual placing.
- Tests for balance and motor coordination (Paper II): rotarod, hanging wire, tail suspension.
- Test for learning and memory (Papers II and III): Morris water maze.
- Tests for emotional reactivity (Paper V): novel cage test (also used in Paper IV), open field, elevated plus-maze, concentric square field, and rat exposure test.

Tests for sensory development

Righting reflex

This test allows the measurement of the postural reflex in mice at an early age. The animal is turned on its back and given a maximum of 30 seconds to return to the upright position. Time of success is recorded.

Postural reflex in an empty cage

This is another test for measuring postural reflex, which can be used for juvenile and adult mice. The animal is placed in an empty cage, which is rapidly moved from side to side and then up and down. The normal postural reflex is to extend all four legs for an upright and balanced position (Crawley & Paylor, 1997).

Nest finding

The nest finding was first developed by Meyerson (1985) to study nest orientation in rats. We used this test to detect the development of olfactory function. Each pup is placed 10 cm away from the nest containing their littermates. Starting from a perpendicular position in relation to the nest, the pup is given a maximum of 60 seconds to find and enter the nest. Time of success was recorded.

Vertical pole

This test measures balance and grip capacity.
The mouse is placed on a pole which is slowly rotated from a horizontal to a vertical position. If the mouse falls before the pole is vertically positioned, the angle of the pole at the time of the fall is recorded.

Visual placing

The mouse is suspended by the tail and lowered towards a surface. A mouse with normal vision will lower its head and suspend the forelimbs before touching the surface with the whiskers.

Tests for balance and motor coordination

Rotarod

The rotarod tests mice for balance and motor coordination, by measuring their ability to stay on a rotating drum at an increasing speed. When mice are placed on the rotating drum, they are forced to run in order to maintain balance. Latency to fall and the maximum speed reached before falling are used as measures of balance and motor coordination.

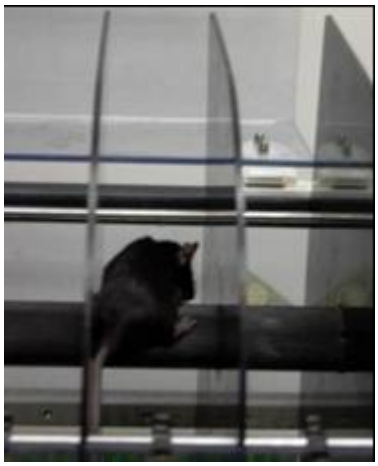

Fig. 1. The rotarod test for balance and motor coordination

Hanging wire

This is a simple test that allows the measurement of grip capacity and muscle strength in mice' forelimbs. The animals are allowed to grab a metal bar with the forelimbs, and the latency to fall is recorded.

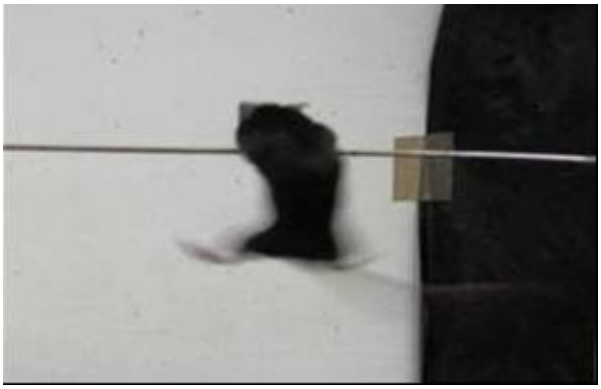

Fig. 2. Hanging wire test for muscular strength and grip capacity

Tail suspension

Holding the mouse by the tail for 30 seconds, allows the detection of clasping (placing the limbs close to the trunk instead of the normal extended position) and grasping (moving the paws as if to grasp something) reflexes in both front and rear paws.

Fig. 3. Tail suspension for detection of clasping/grasping reflex

Tests for learning and memory

Morris watermaze

The watermaze was developed by Morris (1981) as a task to study spatial learning and memory. It was originally used in rats, but it has later been used in mice. It measures the capacity of rodents for spatial learning, through their ability to localize an escape platform placed inside the pool. The platform is submerged and

invisible, therefore mice have to rely on distal cues (e.g. geometric figures hanged on the walls of the room) in order to find it. Learning is measured trough the latency to find the platform, analysis of the swimming pattern and thigmotaxia (tendency to stay close to the walls). The arena for the watermaze is a tank filled with water, and a platform attached to the bottom. The used arenas vary in size, colour and material, as well as the distance to and type of cues used for navigation.

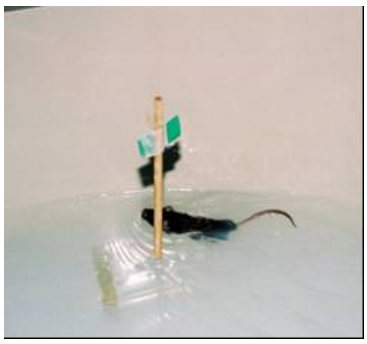

Fig. 4. Morris watermaze for learning and memory. The coloured flag is used to train mice with a visible platform, and is subsequently removed for acquisition trials with invisible platform.

Tests for emotional reactivity

Novel cage test

The novel cage test is used to evaluate emotional reactivity by quantification of exploration and risk assessment behaviour. The mouse is placed in the centre of a clean Makrolon type III cage, and allowed to explore for 5 minutes. Behaviours performed and the use of different locations (latency, frequency of entries and duration of time) are recorded.

Fig. 5. The novel cage test for the assessment of emotional reactivity in pre-weaning mice

Open field

The open field is one of the oldest and most widely used tests for emotional reactivity in rodents (Crawley et al., 1997). It exploits the natural aversion of rodents to exposed areas and allows the evaluation of several ethological parameters which may be used to define the exploratory profile of the animals.
The approach proposed by Hall (1934, cited by Kas & van Ree, 2004) which used decreased ambulation area and increased defecation as indicators of heightened emotional reactivity, has gradually been complemented by over 20 additional behavioural measures (Crawley et al, 1997).

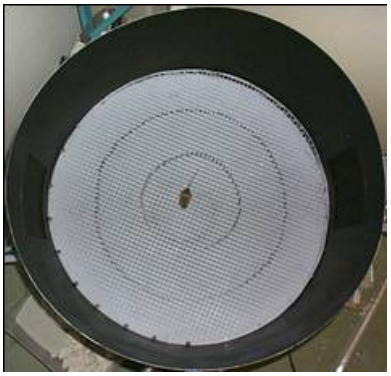

Fig. 6. The arena of the open field used in this thesis.

Elevated plus-maze

The elevated plus-maze is another widely used test for measuring 'anxiety related behaviour' i.e. emotional reactivity. It was developed by Montgomery (1955) and became widely used in pharmacology, since it was shown that administration of anxiogenic drugs causes increased avoidance of the open arms, while anxiolytic drugs cause increased exploration of the open arms (Espejo, 1997). Its anxiogenic component is the exposure to heights and unprotected areas. It is performed in an arena which connects two protected areas (closed arms) with two unprotected areas (open arms) (Fig.7). The animal is placed in the centre of the arena, and exploratory behaviours are recorded, as well as latencies of entry, number of visits and total time spent in each area. The original methods for quantifying emotional reactivity in this test (open arm entries and percent of time spent in open arms) have subsequently been improved through the addition of ethological parameters (e.g. head-dipping and stretched attend posture) (Rodgers et al., 1992).

Fig. 7. The arena of the elevated plus-maze used in this thesis

Concentric square field

The concentric square field was originally established to score functional effects of experimental brain lesions achieved by trauma (Clausen et al., 2001) and microembolization in the rat (Roos et al., 2003), and has previously been used to measure elements of risk and benefit assessment in the exploratory activity of the mouse (Augustsson & Meyerson, 2004, Augustsson et al., 2005). This test is performed by releasing the mouse in the centre of a complex arena (Fig.8), in which it may chose between locations in different environments (e.g. shelter and illumination) (Meyerson et al., 2006). The behaviours performed, as well as latency to enter, number of visits and time spent in each area are recorded.

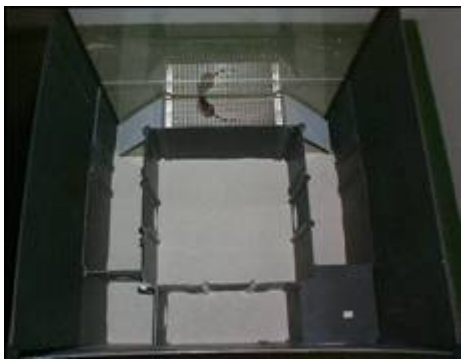

Fig. 8. The arena of the concentric square field

Rat exposure test

The rat exposure test is based on a previous ethological model – the visible burrow system (Blanchard et al., 1995). The main purpose of this test was to elicit and describe risk assessment in mice, and to evaluate the role of risk assessment in defence, by studying subsequent defensive behaviours (Yang et al., 2004). This is done by introducing the mice to the presence of a predator, in an arena which provides the opportunity to explore or to seek shelter (Fig. 9). The rat exposure test has been pharmacologically validated by injecting mice with an anxiolytic

drug which causes reduced risk assessment and avoidance but increased freezing (Blanchard et al., 2003).

Behaviour recordings and scoring

For all behaviour tests video recordings were made by means of digital or analogical cameras, and subsequently data scoring was made manually from the recorded tapes.

Fig. 9. The rat exposure test

Scoring systems

The Observer

This is a widely used, commercially available Windows-based software for behaviour registrations developed by Noldus Information Technology (Wageningen, The Netherlands). It was used, in Paper I, for scoring of behaviours performed and spatial locations.

The VideoMot

The Videomot is a Windows based, commercially available video tracking system, developed by TSE systems (Bad Homburg Germany), which allows recording and analysing animal activity, movement and behaviour, in a wide range of arenas. This software was used in Papers II and III to analyse performance as regards to swimming speed, latency to target and path length as well as time spent in different quadrants in the Morris watermaze.

The Etholog

This is a very simple tool for behaviour scoring, freely available on the web (http://www.geocities.com/CapeCanaveral/Lab/2727/ethohome.html), developed by the University of S. Paulo, Brazil. This software was used in Papers IV and V for scoring of behaviours performed and spatial locations in the novel cage test, open field, elevated plus-maze, concentric square field and rat exposure test.

Experimental procedures

Detailed descriptions of methodologies can be found in the Materials & Methods sections of Papers I – V. This section presents specific comments on the experimental approaches used.

Comments on methodologies

Papers II and III

In paper II different groups of animals were used for each testing age, whereas in paper III the same animals were followed from 6 to 20 months of age. The reason why different animals were used in paper II was to avoid possible carry-over effects of habituation on the results. In paper III however, given the experimental design with six combinations of genotype and housing environment, we considered that the use of different groups of animals per age would have caused an unacceptable increase in the number of animals needed. We therefore opted for a repeated testing approach, as a means of reduction.

In both Paper II and Paper III, the selected protocol for the Morris watermaze was based on the previously existing literature for learning and memory assessment in mice (e.g. Crawley, 2000). However, this protocol was observed to be quite stressful, as after each daily set of trials, mice were observed to spend a considerable amount of time lying immobile or shaking when returned to the home cage (author's own observation), although the home cage was placed close to a heating unit. The watermaze for mice was originally developed for rats. Due to the differences in biology and physiology between mice and rats, previous studies pointed out the need to develop specific tests and/or protocols for mice (e. g. Wishaw & Tomie, 1996). In the wild, most strains of rats live close to the water, with swimming being part of their normal behaviour repertoire. On the other hand, most strains of mice do not swim in the wild, thus swimming may be stressful and induce hypothermia (Iivonen et al., 2003). Therefore watermaze protocols for mice should include intertrial intervals which allow to recover normal body temperature. In fact, in unpublished studies recently performed by the author, a protocol was used which included intertrial intervals of 10 to 15 minutes. When using this recovery period no signs of immobility and shaking were observed in the tested mice. They simply engaged in grooming and explorative behaviour. With the protocol used in Papers II and III, some mice might have developed hypothermia which might have affected the results obtained.

Paper IV

In addition to the methodological study presented in Paper IV, a preliminary study was conducted by screening two genetically modified lines and one spontaneous mutant. The monitored lines were:

The Nestin PDGF B mouse

Transgenic mice carrying the human PDGF-B and the LacZ reporter gene under control of the human nestin enhancer were generated by the oocyte injection technique at Uppsala University Transgenic Facility (Enarsson et al, submitted manuscript).

The GAD67- GFP knock-in mouse

The glutamic acid decarboxylase (GAD) catalyzes the decarboxylation reaction from which glutamic acid originates gamma aminobutyric acid (GABA), which is a major inhibitory neurotransmitter in the brain. GAD67 is responsible for the

synthesis of 90% of GABA in the brain (Soghomonian & Martin, 1998). The GAD67-GFP knock-in mouse has inserted cDNA which encodes the enhanced green fluorescent protein, in the GAD67 gene, and was developed to study the distribution, morphology, electrophysiologic properties and development of GABAergic neurons (Tamamaki et al., 2003).

The Kv1.1 null mouse

These mice have a spontaneous mutation which causes deficiency of the voltage-gated potassium channel alpha subunit K(V)1.1, associated with the display of frequent spontaneous seizures throughout adult life (Smart et al., 1998). The loss of K(V)1.1 from its normal localization in axons and terminals of the CA3 region results in increased excitability in the CA3 recurrent axon collateral system, contributing to the limbic and tonic-clonic components of the observed epileptic phenotype (Zhang et al., 1999, Zhou et al., 1999).

Paper V

In Paper V the rat exposure test was included in the test battery for emotional reactivity. The use of this test is usually accompanied by the injection of amphetamines, in the rats that serve as stimulus, to standardize their behaviour. However, in Paper V, this was not done due to both ethical considerations and to the fact that the administration of amphetamines will modify the natural exploratory and predatory behaviour of the rats. This resulted in increased variability of the behaviour of stimuli rats, and to balance that effect, the number of mice which were assigned to each rat was balanced by strain and sex.

Statistical analysis

In general, the approach for statistical analysis was to test raw data for distribution using the Kolmogorov-Smirnov test and for homogeneity of variances using the Levene test. For data that followed a normal distribution, either in raw data or after transformation, analysis of variance (ANOVA) was used, followed by post-hoc multiple comparisons, using Bonferroni test. If the conditions for parametric analysis were not met, Kruskall-Wallis test was used followed by post-hoc multiple comparisons using Mann-Whitney U test. In this case, Bonferroni corrections were applied to multiple comparisons, by multiplying the obtained p-value by the total number of comparisons. In all papers differences were considered significant at $p<0.05$ level. Unless stated otherwise, all statistics were carried out using SPSS v14.0 for Windows.

In Papers II and III General Linear Model Repeated Measures was used for parametric data. If data did not conform to the assumptions for parametric analysis, Generalized Linear Model was used.

In Paper IV, categorical data (0/1, yes/no) was analysed using Pearson's Chi-square, which is based on the comparison between the frequencies that are observed for each category, with the frequencies that would be expected by chance.

In Paper V, statistical analysis was supplemented with a multivariate data analysis using a pattern recognition based approach, the SIMCA (Soft Independent

Modelling of Class Analogy) principal components analysis. This analysis was carried out using the PCA SIMCA-P+11 software from Umetrics (http://www.umetrics.com).

Results and comments

Impact of rearing environment on behaviour in adulthood (Paper I)

No persistent effects were found on adult home cage behaviour, caused by the introduction of environmental enrichment during the pre-weaning period. A significant increase on home cage activity was found at four weeks of age for mice that changed from furnished to standard cages, but this effect was only temporary, as at 8 weeks of age no such difference was found. Postweaning environmental enrichment caused a decrease in feeding behaviour, self grooming and allogrooming and a sex difference was found with more exploratory behaviours and higher occurrence of stereotypies in female mice.

Comments

Mouse pups are poorly developed at birth and they spend the first 2 weeks of life inside the nest, where they are totally dependent on the mother (Latham & Mason, 2004). Therefore, the effects of the early postnatal period on adult behaviour are to a larger extent affected by maternal care rather than by the physical environment in the home cage. The sensory mechanisms that underlie adult-like behaviour (e.g. hearing, vision) only start to develop after day 10-12 (Fox, 1965), which is also the time when mice start expressing social behaviour, and start exploring and leaving the nest (Latham and Mason, 2004). Thus, the pre-weaning period in which pups interact directly with the cage environment, is restricted to around 10 days. That is probably a too short time period to affect and modulate adult behaviour. On the other hand, an increase in activity was found at 4 weeks of age for mice that changed from enriched to standard cages. This may indicate attempts to escape, as well as a decrease in resting time due to the absence of nesting material.

The higher performance of grooming in mice kept in standard cages may be related to the performance of barbering and whisker trimming. These two behaviour responses are frequently observed at the IBMC animal facility and have also previously been reported commonly occur in C57BL/6 mice (Sarna et al., 2000, Garner et al., 2004) with a higher incidence in female mice (Garner et al., 2004). This could not be verified in the present study, since the mice used in this experiment were euthanized at 9 weeks of age, and barbering is known to develop later in age (Garner et al, 2004).

Characterization of the *leaner* heterozygous mouse (Papers II and III)

In Paper II, evidence of motor and cognitive impairments was found in the *leaner* heterozygous mouse. Motor impairments were found through the detection of the clasping reflex, which occurred in all *leaner* mice from 6 months of age, whereas in wild type mice this was only present in 17% of the mice at 12 months of age and in 50% of the mice at 22 months of age. A motor impairment was also found in the rotarod test, where *leaner* mice showed impaired performance when compared to wild type mice in the more demanding protocol performed at 12 months of age.
In both Papers II and III, *leaner* heterozygous mice showed impaired performance in the Morris watermaze. At 6 months of age *leaner* mice showed a higher latency to target, a longer pathlength and a lower swim speed when compared to wild type mice. At 20 – 22 months of age *leaner* mice showed a longer pathlength and latency to target than wild type mice. Only wild type animals showed evidence of remembering the platform location in the probe test (which tests memory retention when the escape platform is removed). Additionally, in the presence of a distractor cue, *leaner* mice presented a lower swim speed and in a task for non-spatial learning they had a longer pathlength than wild type mice.

Comments

In the rotarod test significant differences were only evident when a more demanding protocol was used, highlighting the importance of establishing the optimal approach for each particular study, which can be facilitated by the performance of pilots studies, as well as by sharing experiences across laboratories.
In Paper II, different groups of mutant and control animals were used for each age, whereas in Paper III the same animals were followed from 6 to 20 months of age. The fact that overall both studies produced consistent results, supports the argument for the use of repeated testing. Animals can be re-used over time resulting in reliable data (Sven Ove Ögren, personal communication), but re-use must be weighed against the stress, pain or discomfort associated with each specific test. Repeated testing on the same animals may be beneficial by minimizing experimental variations, therefore improving the validity of results while meeting the criteria for Reduction of the number of animals needed.

Effect of environmental enrichment on the cognitive performance of wild type and *leaner* mice (Paper III)

Environmental enrichment enhanced the performance in the Morris watermaze, regarding the latency to target and the pathlength, during training (visible platform) and acquisition (invisible platform), and time spent in the target quadrant during retention (platform removed). During training at 6 months of age, the latency to target was lower in mice from furnished cages than in mice from standard and barren cages and during acquisition. At both 6 and 20 months of age

latency to target was lower in mice from furnished and standard cages when compared to mice from barren cages.
At 6 and 12 months of age during training, the pathlength was shorter in mice from furnished and standard cages than in mice from barren cages, and during acquisition it was shorter in mice from furnished cages when compared to both barren and standard cages. At 20 months of age for acquisition, the pathlength was shorter in mice from both standard and furnished cages when compared to mice housed in barren cages.
During retention, at 6 months of age, mice housed in furnished cages spent a greater percentage of time swimming in the target quadrant than animals from standard and barren environments. In the probe test conducted on the first testing day at 20 months of age, mice in furnished and standard cages spent a higher percentage of time swimming in the target quadrant than mice in barren cages.
At 6 months of age mice from all environments showed evidence of remembering the platform location. At both 12 and 20 months of age this was only found for mice from furnished environments.
No significant interactions between genotype and housing environment were found, indicating that the effects of environmental enrichment were similar for both wild type and *leaner* mice.

Comments

Overall, the use of environmental enrichment enhanced the performance in the Morris water maze for both wild type and *leaner* heterozygous. The fact that for some of the parameters measured there was no difference between the performance of mice in furnished and standard cages – shorter latency to target during acquisition at 6 and 20 months of age and shorter path length during acquisition at 20 months of age - indicates that the simple provision of nesting material and a cardboard tube (introducing the possibility to build a nest and to seek shelter) is sufficient to cause some degree of improvement of the cognitive functions.

Routine characterization of mutant mice during the preweaning period (Paper IV)

Wild type mice (Paper IV)

Phenotype monitoring through the application of the developed score-sheets allowed the detection of strain/genotype differences in the occurrence of vocalizations, passivity and provoked biting, as well as in morphological development (body weight gain), sensory development (righting reflex and nest finding tests) and emotional reactivity. The number of C57BL/6 mice performing the righting reflex test successfully on day 3 was lower when compared to the other 3 strains. By day 4, all 129S6, BALB/C and hybrid pups had succeeded the test, while some C57BL/6 pups only succeeded on day 5. In the nest finding test, most 129S6 and C57BL/6 mice performed the test successfully on day 7, differing significantly from both BALB/C and hybrid pups. For BALB/C and hybrid pups

there was a percentage of mice that never succeeded in the test, whereas both 129S6 and C57BL/6 had all succeeded by day 9.
Differences in emotional reactivity were found through evaluation of exploration and risk assessment in the novel cage test. Overall, 129S6 and BALB/C mice showed a higher emotional reactivity than C57BL/6 and hybrid mice, displaying less locomotion and exploratory behaviours and higher levels of stretched approaches and stretched attend postures.

Preliminary results from some strains of mutant mice

Monitoring of mutant litters allowed the detection of individual cases that presented differences in development and/or behaviour, when compared to littermates (see Appendix A). The most commonly found problems were absence of milk spots, abnormal body weight, passivity, delayed development of the fur and delayed opening of the eyes and ears. Altered physiological parameters were found at the clinical examination performed at weaning, including a bad condition of the eyes (presence of secretions), trimmed whiskers and absence of response to touch or sound stimulation. In the case of the Kv1.1 null mouse, several pups presented abnormal gait, body and tail posture, dehydration, altered respiratory rate, abnormal tonus of the abdomen and limbs, and failure to perform the postural reflex and vertical pole tests successfully.

Comments

Application of the developed score-sheet allowed the detection of strain/genotype differences in development and behaviour profiles, as well as the detection of several individual cases presenting deviant/abnormal parameters of development. Although the current form of this protocol can be successfully used, there is room for improvement, in particular concerning the time it takes to apply it. The nest finding test was not effective, even when performed in a clean cage. Only a small percentage of mice performed the test successfully on day 7, and some mice never succeeded in the test. An alternative test should therefore be developed for the assessment of olfactory function. A suggestion for a possible alternative is to evaluate it during the clinical examination, by presenting an aversive odour close to the mouse's nose and registering the presence/absence of an aversive reaction.

Preweaning assessment of emotional reactivity (Paper V)

Assessment of emotional reactivity at weaning with the novel cage test, was found to be an effective predictor for emotional reactivity in adult mice. There was a high correspondence between the results obtained by each test, indicating that the novel cage test is as efficient as established tests for the detection of differences in emotional reactivity and that the exploratory and risk assessment profile of the mouse is already developed at weaning. Figures 10 -12 illustrate the performance of 3 different tests carried out at different ages – novel cage test (weaning: 19 – 21 days of age), open field (adolescence: 5 weeks of age) and elevated plus-maze (adulthood: 9 weeks of age), showing the correspondence of results between tests and across ages. The PCA charts show the grouping of the mouse strains

according to the behaviours performed in each test. The score plots (figs. 10a-12a) show that for all tests 129S6 mice are clearly separated from C57BL/6 and the loading plots (figs. 10b-12b) show that for all tests 129S6 are grouped by the performance of stretched attends, stretched approaches, and immobility, whereas C57BL/6 were grouped by the performance of walk and wall rearing. In the open field the performance of grooming by 129S6 mice also contributed to the separation of this strain. Hybrids and C57BL/6 were generally grouped by the performance of locomotion and wall rearing, but separated by the performance of unprotected head dips in the elevated plus-maze.

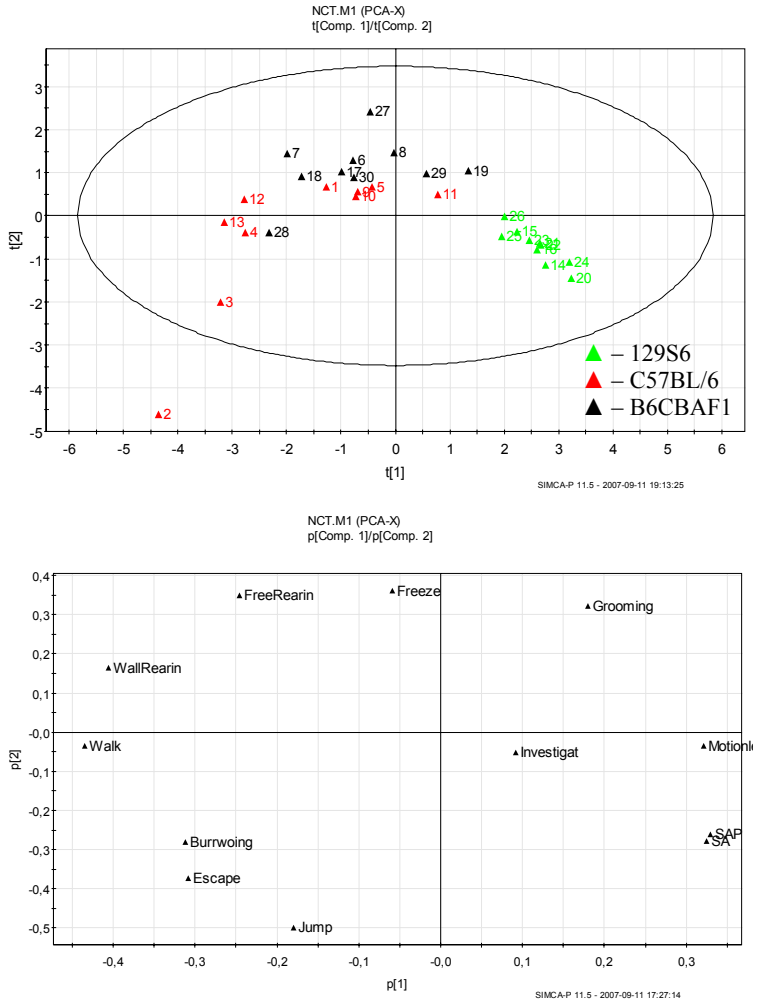

Fig. 10. PCA-SIMCA analysis for the novel cage test

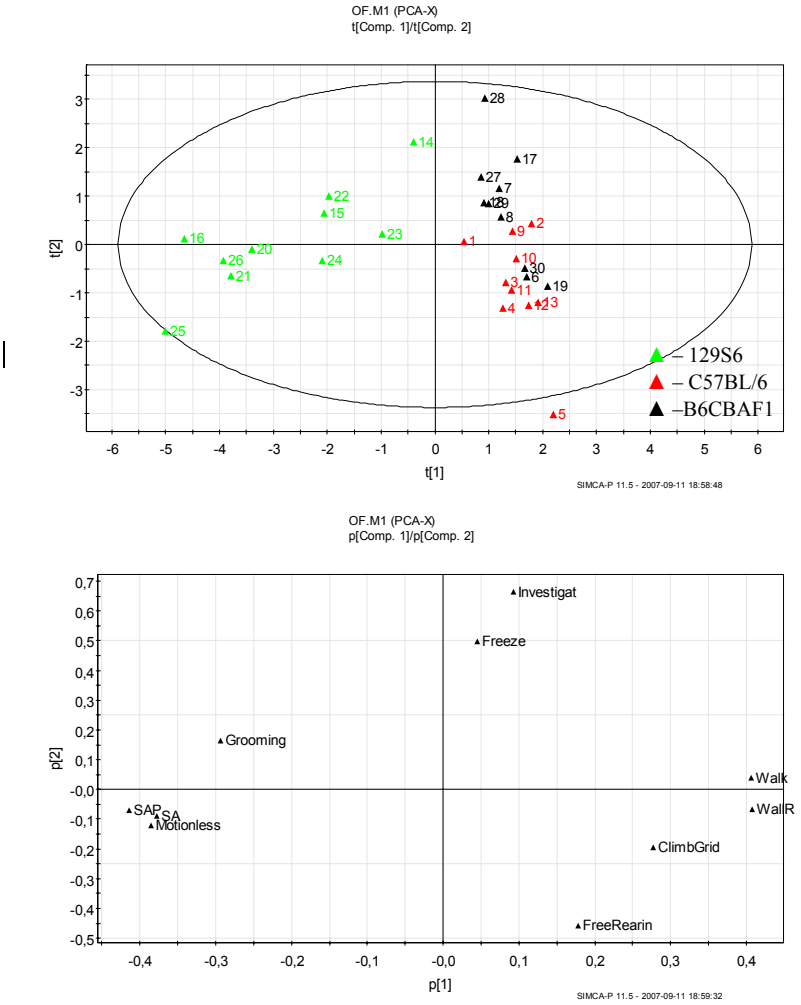

Fig. 11. PCA-SIMCA analysis for the open field test

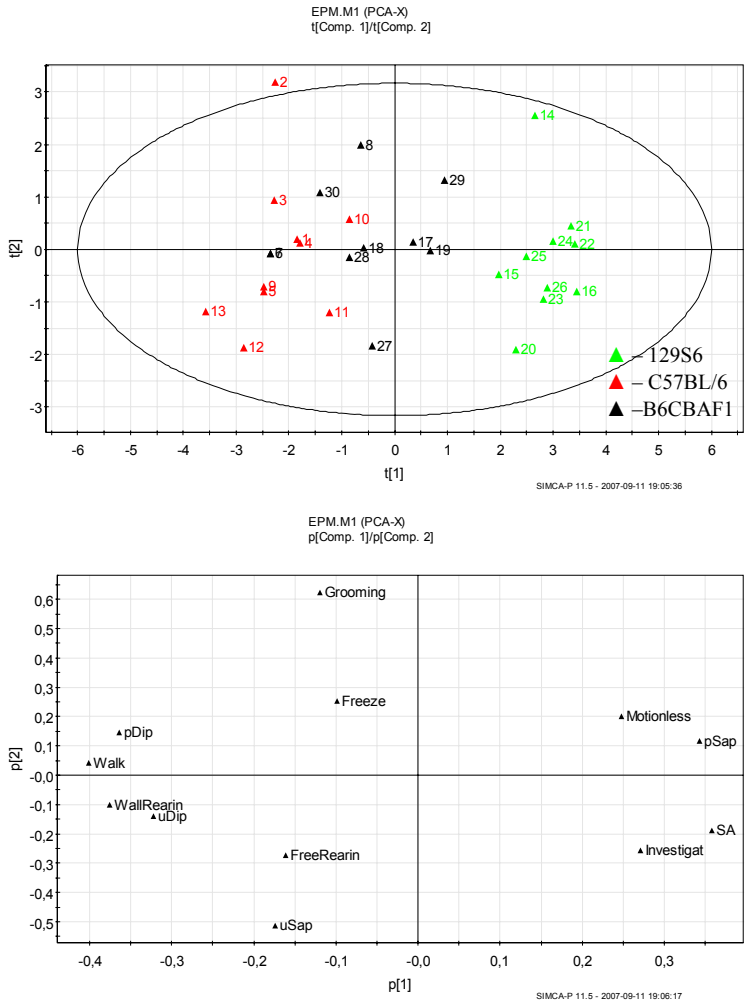

Fig.12. PCA-SIMCA analysis for the elevated plus-maze

Comments

Although the novel cage test was found to provide data which were consistent with other more established tests, mice of all strains spent a higher percentage of time performing selfgrooming behaviour. At weaning, the novel cage test was performed after the clinical examination, which involves handling and restraining of the mice. It is therefore possible that the higher performance of grooming observed at this age was more related to the restraining than to an effect of the test itself. In the future, the novel cage test should be carried out prior to the clinical examination to avoid possible effects of handling in the results obtained.

General discussion

Introduction of environmental enrichment

From a Refinement and welfare perspective, environmental enrichment should be implemented as a means to give the animals a chance to express their natural behaviours, which are limited in a non-enriched cage. Environmental enrichment during the pre-weaning period has been reported to affect several aspects of rodent behaviour. This is thought to be at least partly mediated by maternal care. However, in this thesis no major or permanent effects of the rearing environment were found on the home cage behaviour of mice during adulthood. Home cage behaviour observations have previously been used in studies aiming to investigate voluntary exercise (De Visser et al., 2005), environmental novelty (De Visser et al., 2007) and maternal behaviour (Pedersen et al., 2006), and Harri et al (1999) and Nevison et al., (1999) (cited by Olsson & Dahlborn) both studied time budgets. It is a useful methodology to understand how several factors affect behaviour in captivity. However, such observations should be complemented by more specific behaviour tests to investigate parameters such as emotional reactivity, social behaviour and cognitive performance and eventually complementing those tests with physiological measurements. At present, there is no information regarding how environmental enrichment affects maternal care in terms of time spent in the nest, nursing and grooming the pups (Weber & Olsson, Accepted for publication). Future studies should focus on these aspects in order to provide more information about how maternal care influences adult behaviour.

The provision of environmental enrichment to the *leaner* heterozygous mouse improved spatial learning and memory. Previous studies on brain plasticity have reported effects at the cognitive level, through anatomical and physiological changes in the brain. Introduction of environmental enrichment which diversifies behaviour, will probably turn mice into better models of research by making their behaviour and physiology more representative of that of the normal functioning of their species or of humans (Garner, 2005). Besides the influence of environmental enrichment on the brain morphology, it has also been reported to cause physiological changes and improved recovery from injury, disease and brain lesions. By affecting the health and survival of mouse disease models, it will also have a positive effect on mouse welfare. Studying the development of a disease in both standard and enriched cages, may therefore add valuable information when compared to executing the experiment in only one environment. This will in turn improve research quality. Results from the previous studies on the effects of environmental enrichment on the behaviour of mice, showed that some items are of great importance (e.g. nesting material), while indicating that some enrichment strategies may have inherent risks (e.g. increasing cage complexity for male mice). A European Union Recommendation was recently presented - 2007/526/EC - on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes, which requires that all small rodents are given nesting material (European Union, 2007).

Mouse phenotyping and welfare monitoring

Several mouse spontaneous mutants have been useful for the study of gene functions and disease mechanisms (e.g. Lurcher, Stargazer and Woozy mice (see Sidman et al., 1965 for descriptions)). Phenotyping of mouse spontaneous mutants can therefore provide valuable information regarding behaviour alterations which have not previously been described. The clinical conditions and symptoms described in humans provide a good starting point for the detection and characterization of altered phenotypes, while a correct establishment of testing protocols will allow the detection of subtle impairments. The existing protocols for characterization allowed the phenotyping of many genetically modified mice, and a subsequent listing of behavioural phenotypes in accessible databases (e.g. Bolivar et al., 2000, http://biomednet.com/db/mkmd and http://tbase.jax.org). This allows researchers to determine the possible usefulness of specific mouse lines. Similar information does not exist concerning possible welfare problems presented by mutant mice. Early monitoring is therefore important to predict severe phenotypes and assure a humane use of animals, as well as to address the impact of unexpected phenotypes on experimental results. Such a routine was developed and evaluated as part of this thesis. Its application allowed the detection of cases of mice with abnormal development, which could mean an impaired welfare and also influence the experimental outcome. The presented routine was easy to learn, by researchers, technicians and students, and for a better comprehension of the parameters involved and the methods for scoring, an educational CD was developed which can be made available. The implementation of this routine in production and breeding facilities, where large numbers of new transgenic mouse strains are produced, will represent an increase in the workload which demands extra funds and time. It is important that such resources are made available since the number of mice with genetic modifications is increasing. Detection of unexpected effects on development and welfare is important for the improvement of research quality through the recognition and elimination of potential confounding factors.

A suggestion for application of this characterization routine would be to follow the first 10 litters when establishing a new line, and using wild type littermates as controls. If the physiological or behavioural development is deviated/abnormal, the investigator and veterinarian should be contacted. If a severe phenotype is detected, an immediate action for improved care should be initiated and the phenotype should be further analysed in detail. The results should then be reported to the ethical committee to decide on the scientific value of this particular genetic modification versus the degree of animal suffering.

Conclusions

- This thesis shows that refinement of mouse husbandry through the implementation of cage enrichment and routines for welfare monitoring in mutant mice can improve animal welfare and research quality.

- The beneficial effects of environmental enrichment on cognitive abilities found in several animal models can now be extended to the *leaner* heterozygous mouse, where it was found to enhance spatial learning and memory performance.

- Phenotyping protocols have provided knowledge about the behavioural characteristics of many genetically modified mouse lines, as well as of several spontaneous mutants. The *leaner* heterozygous, previously described as normal, is now known to present motor and cognitive impairments when studied systematically. These results highlight the importance of characterizing spontaneous mutants where the same mutations are known to cause clinical symptoms in humans, and also to refine testing protocols so that more subtle alterations can be detected.

- Characterization of mutant mice should be extended to the screening of welfare problems, by application of routines for welfare monitoring and defined criteria for humane endpoints. The monitoring routine proposed in this thesis was effective in detecting several developmental deviations/abnormalities which are likely to compromise welfare and survival.

Scope for future research

In this thesis, environmental enrichment provided during the pre-weaning period did not have permanent effects in mice home cage behaviour during adulthood. Further studies focused on the influence of pre-weaning environmental enrichment on maternal care followed by behavioural testing of the litters as adults, could shed light on the modulation of mouse behaviour by maternal care.

The routine characterization presented in this thesis was effective in detecting developmental differences between strains/genotypes, and results from a preliminary study show that it can detect possible welfare problems occurring in genetically modified mice. This routine should be further validated by applying it to several newly developed lines of genetically modified animals.

The application of the novel cage test at weaning showed a consistency of results when compared to other tests performed during adolescence and adulthood. Future studies will address the development of a score-sheet for real-time scorings, and compare its efficacy with that obtained from scoring of film recordings.

Resumo para divulgação científica

Hoje em dia o ratinho[1] é o mamífero mais utilizado em investigação. Isto deve-se não só ao facto de ser uma espécie fácil de criar e manter num laboratório, mas principalmente devido á possibilidade de manipular o seu genoma, constituindo assim um importante recurso para a investigação biomédica. Avanços na área da engenharia genética, permitiram a criação de inúmeras linhas de animais transgénicos que permitem estudar os mecanismos de várias doenças humanas, bem como compreender a função e expressão de muitos genes. Não é por isso surpreendente que o uso do ratinho em investigação, tenha vindo a aumentar, em particular na última década, e que continue a aumentar num futuro próximo.

Para encorajar o uso de alternativas aos animais e promover métodos de investigação mais humanos, i.e. que diminuam o sofrimento dos animais e promovam o seu bem-estar, os investigadores William Russell e Rex Burch, no final da década de 50 propuseram o príncipio dos 3 R's - Substituição (em inglês 'replacement'), Redução e Refinamento, que é hoje em dia vastamente referido pelas comissões de ética animal e pela legislação de alguns países.
Segundo o princípio dos 3 R's o uso de animais em experimentação deve ser evitado sempre que haja uma alternativa disponível (substituição), se não houver alternativa, o número de animais usado deve ser o mínimo necessário para obter resultados conclusivos (redução), e as técnicas experimentais devem garantir o mínimo possível de dor e de stress (refinamento).

A presente tese, focada no terceiro 'R' – refinamento, discute duas importantes técnicas utilizadas nesse contexto – o uso de enriquecimento ambiental, e a caracterização de ratinhos geneticamente modificados. Os principais objectivos subjacentes a este trabalho são:

- Estudar o efeito do enriquecimento ambiental no comportamento do ratinho.

- Utilizar um conjunto de testes comportamentais para caracterizar um ratinho mutante, que é usado como modelo de doenças neurodegenerativas em humanos.

- Desenvolver uma rotina para a caracterização de novas linhas de ratinhos mutantes/geneticamente modificados, durante o período entre o nascimento e o desmame, focada na avaliação do bem-estar dos animais.

[1] Nota: embora em Português corrente usemos as palavras rato e ratazana, para nos referirmos ás espécies *Mus musculu*s e *Ratus norvegicus*, respectivamente, em 'linguagem de laboratório' o rato (*Mus musculus*) é referido como ratinho (ou murganho), enquanto á ratazana (*Ratus norvegicus*) se chama rato.

- Validar um teste para a avaliação de reactividade emocional, incluído no protocolo de caracterização desenvolvido.

O Enriquecimento Ambiental

A expressão 'enriquecimento ambiental' refere-se á modificação do ambiente onde são alojados os ratinhos - em geral caixas de plástico transparente com um tamanho que varia entre 400 e 800 cm^2 - de modo a torná-lo mais complexo e com mais oportunidades de expressar o seu comportamento natural.
O interesse na introdução de enriquecimento ambiental para os animais de laboratório começou com o cientista Donald Hebb, em finais da década de 40. Hebb constatou que os ratos que mantinha em casa como animais de estimação, tinham maior facilidade em resolver tarefas que exigiam aprendizagem e memória, do que os ratos que mantinha no seu laboratório. Estas observações marcaram o início de inúmeras experiências em laboratório, em que diversos investigadores descobriram que o aumento da complexidade do ambiente pode influenciar a morfologia e plasticidade do cérebro, mesmo durante a idade adulta. Estas descobertas foram muito importantes, pois abriram caminho para o estudo de possíveis efeitos do enriquecimento ambiental em várias doenças neurodegenerativas humanas. Foi de facto descoberto que o recurso a um ambiente mais complexo é benéfico para algumas doenças, como Parkinson, Alzheimer e Huntington, nas quais atrasa o início e progresso dos sintomas (em modelos animais).
Durante a década de 90, o enriquecimento ambiental começou a ser utilizado em muitos laboratórios como método de promover o bem-estar dos animais usados em investigação. O alojamento em grupos (enriquecimento social), aumento do tamanho das caixas e introdução de vários objectos nas caixas (enriquecimento físico), tais como material de ninho, casotas, e tubos de cartão, possibilita que os ratinhos possam expressar alguns dos seus comportamentos naturais como constituir uma hierarquia social, construir um ninho, ou procurar abrigo. Para além dos efeitos que tem no cérebro, este aumento de complexidade e 'naturalidade' do ambiente traduz-se numa redução do stress e desconforto associados ao cativeiro, o que por sua vez torna o ratinho num melhor modelo para investigação. Tal como afirmou o investigador Trevor Poole, '*Animais felizes fazem boa ciência*'.

Caracterização de ratinhos geneticamente modificados

Com o aumento no uso de ratinhos mutantes/geneticamente modificados, o recurso a técnicas de caracterização desses animais é de grande importância, para entender de que modo a mutação ou alteração genética se traduz numa alteração do fenótipo. Diversos conjuntos de testes comportamentais estão disponíveis para esse efeito. No entanto, os protocolos de caracterização existentes foram concebidos para utilizar durante a idade adulta e são principalmente úteis para estudar aspectos com interesse contextuado numa determinada experiência. A detecção de alguns casos de modificações genéticas que levaram a uma diminuição no bem-estar dos animais, levou alguns cientistas a proporem a necessidade de se desenvolverem protocolos de caracterização mais focados na detecção de problemas de bem-estar, que podem exigir cuidados especiais e/ou a

eutanásia. Os ratinhos nascem pouco desenvolvidos, tanto do ponto de vista morfológico, como neurológico/sensorial. Durante as primeiras 3 semanas de vida, o seu desenvolvimento é extremamente rápido. É por isso importante a caracterização dos animais durante este período de desenvolvimento, de modo a detectar possíveis atrasos ou anormalidades no desenvolvimento, que poderão ter influência tanto no bem-estar dos animais como em futuros resultados experimentais, e que poderão já não ser detectáveis durante a vida adulta.

Reactividade emocional

Embora sejamos capazes de reconhecer uma emoção em nós próprios, encontrar uma definição para explicar o que entendemos como emoção não é uma tarefa simples. Referimo-nos frequentemente a expressões como 'sentimento' e 'disposição' (boa/má), para expressar um processo emocional. Por outro lado, a quantificação de uma emoção é ainda mais difícil, e para podermos avaliar uma emoção noutra pessoa, baseamo-nos na sua própria descrição das suas sensações. Nos animais, processos emocionais relacionados com o medo ou a dor, por exemplo, também existem e exercem uma função importante para a sua sobrevivência. Avaliar emoções num animal é ainda mais difícil, uma vez que este não pode recorrer ao discurso verbal para nos explicar o que sente. Uma possível definição para emoção seria 'um estado mental que se manifesta através de um certo número de processos fisiológicos e psicológicos (comportamentais)'. A avaliação de uma emoção pode portanto ser efectuada através do estudo de alterações na fisiologia e/ou do comportamento, ao qual se dá o nome de 'reactividade emocional'.

Efeitos do enriquecimento ambiental durante o período pré-desmame no comportamento do ratinho

O primeiro estudo desta tese avaliou os efeitos do enriquecimento ambiental durante o período pré-desmame, no comportamento do ratinho. O comportamento dos ratinhos na caixa de alojamento foi registado ás 4 e 8 semanas de vida. Os resultados deste estudo indicam que a introdução de enriquecimento durante o período pré-desmame não causa alterações permanentes no comportamento do ratinho em adulto. Uma possível explicação para esta ausência de efeito, é que nas primeiras duas semanas de vida, o ratinho recém-nascido passa a maior parte do tempo no ninho, sob os cuidados da mãe, e por isso não interage com o ambiente físico da caixa. O desmame é efectuado 21 dias após o nascimento, o que deixa apenas 7 a 10 dias para exploração da caixa, sendo provavelmente um período demasiado curto para exercer efeitos marcados sobre o comportamento.

Caracterização fenotípica do ratinho heterozigótico para a mutação espontânea *leaner*

A mutação espontânea *leaner* foi descrita pela primeira vez em ratinhos na década de 60. Esta mutação afecta um canal de cálcio, e em ratinhos homozigóticos causa sérios problemas ao nível da capacidade motora desde os primeiros dias de vida, e

estes animais acabam por morrer até ás 3 semanas de idade. No entanto, os ratinhos heterozigóticos têm sido descritos como normais. A diversidade de sintomas que ocorrem em humanos com um tipo de mutação semelhante e a diversidade de funções que o cálcio exerce no nosso cérebro, geraram a hipótese de que os ratinhos heterozigóticos poderiam apresentar alterações fenotípicas subtis e/ou ocorrendo numa idade mais tardia. Um conjunto de testes comportamentais usado aos 6, 12 e 22 meses de idade, e que incluiu testes de coordenação motora e testes de aprendizagem e memória, revelou que de facto ratinhos heterozigóticos apresentam uma redução das capacidades motora e cognitiva (aprendizagem e memória). Estes resultados têm grande importância para o uso destes ratinhos como modelo animal de doenças neurodegenerativas em humanos.

Efeito do enriquecimento ambiental na capacidade cognitiva do ratinho heterozigótico *leaner*

Num estudo subsequente, grupos de ratinhos heterozigóticos (e controlos), foram alojados em caixas com 3 níveis diferentes de complexidade: ambiente empobrecido: ausência de objectos; ambiente standard: presença de material de ninho e um rolo de cartão; ambiente enriquecido: caixa com o dobro do tamanho, e com casota, tubo de cartão, material de ninho, roda para correr, e um objecto extra, trocado semanalmente de modo a introduzir um elemento de novidade.
A capacidade de aprendizagem e memória destes animais foi testada aos 6, 12 e 20 meses de idade num 'labirinto de água' - um teste desenvolvido por Richard Morris ('Morris water maze'). Neste teste os ratinhos são colocados dentro de um tanque com água e para sairem da água têm nadar e localizar uma plataforma escondida, com a ajuda de pistas distais (ex. figuras geométricas coladas na parede), ou seja, este labirinto testa a capacidade de um ratinho aprender a orientar-se no espaço (tal como nós, ao usarmos pontos de referência para chegar a um determinado local), e a sua capacidade para memorizar aquilo que aprenderam.
Os resultados deste estudo mostraram que tanto os ratinhos heterozigóticos como os controlos, melhoram a sua capacidade de aprendizagem quando alojados num ambiente enriquecido. Em alguns dos parâmetros medidos, animais dos ambientes standard e enriquecido tiveram uma performance semelhante, o que indica que a simples introdução de oportunidades para construir um ninho e para procurar esconderijos é suficiente para causar um certo grau de melhoria das capacidades cognitivas.

Caracterização fenotípica e bem-estar animal

Com vista a monitorizar o bem-estar animal em novas linhas de ratinhos geneticamente modificados, foi desenvolvido um protocolo para integração nas rotinas de maneio durante o período pré-desmame. Este protocolo inclui a avaliação de vários parâmetros relativos ao desenvolvimento físico, bem como vários testes comportamentais para avaliar o desenvolvimento neurológico. No dia do desmame procede-se a um exame clínico para avaliar a condição física e testar reflexos.

Os resultados deste estudo, mostraram que o protocolo desenvolvido é eficiente na detecção de desvios/anormalidades no desenvolvimento do ratinho, bem como na detecção de casos que afectam o bem-estar dos animais, e que exigem a aplicação de cuidados especiais e/ou a eutanásia.

Avaliação da reactividade emocional ao desmame

O exame clínico efectuado no protocolo desenvolvido, inclui um teste para a avaliação da reactividade emocional, cujo desenvolvimento foi feito no âmbito da presente tese – o 'novel cage test'. Este teste consiste em colocar o ratinho no centro de uma caixa semelhante ás caixas de alojamento, mas que está limpa e vazia – introduzindo, portanto, como elementos de novidade, a ausência de cheiros familiares e dos objectos que estão normalmente dentro da caixa de alojamento (ex. casota e material de ninho), e deixá-lo explorar a caixa por 5 minutos, registando os comportamentos efectuados. Para avaliar a eficiência deste teste, três grupos de ratinhos de estirpes diferentes, previamente descritas como tendo diferente reactividade emocional, foram testados no novel cage test, e em 4 outros testes previamente validados para testar a reactividade emocional em animais adultos – o 'open field', o 'elevated plus-maze', o 'concentric square field' e o 'rat exposure test'. Os resultados deste estudo mostraram uma consistência em relação aos comportamentos exibidos, bem como em relação ao uso das diferentes áreas nas arenas de teste. Esta consistência indica não só que o 'novel cage test' é eficiente para a avaliação da reactividade emocional, mas também que nos ratinhos esse parâmetro está em grande medida desenvolvido ás 3 semanas de idade, e que pode portanto ser avaliado desde bastante cedo.

Conclusões

A introdução de enriquecimento ambiental é importante não só para permitir a expressão de comportamentos naturais, mas também para ajudar a compreender os mecanismos associados a doenças neurodegenerativas em modelos animais.

A implementação de rotinas para a caracterização de novas linhas de animais geneticamente modificados, permite a melhoria do bem-estar animal através da detecção de casos que exijam cuidados especiais ou eutanásia, e contribui para melhorar a qualidade dos resultados experimentais, através da diminuição de factores com impacto nas experiências efectuadas (ex. dor, subdesenvolvimento, reactividade emocional alterada).

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Appendix A.

Table A1. Number of mouse pups found with development deviations between postnatal day 1 and postnatal day 14.

	Nestin PDGF B		GAD67-GFP		Kv1.1		
	Wild type n=55	Trans-genic n=43	Wild type n=33	Trans-genic n=22	Wild type n=18	Hetero zyg. n=32	Homo zyg n=13
Day 1							
Out of nest	0	0	1	2	0	0	0
Weight	1↓	0	0	0	0	0	0
Absence of milk spot	0	0	1	0	0	2	3
Passive	0	0	0	0	1	2	1
Vocalization	28	19	31	21	2	3	5
Day 3							
Dead	0	0	0	0	*	*	*
Weight	1↓	1↓	0	1↓	0	0	0
Absence of milk spot	0	0	1	1	0	0	0
Passive					2	7	2
Vocalization	28	25	24	17	10	15	6
Day 7							
Dead	0	0	0	0	0	0	0
Weight	1↓	1↓	0	1↓	0	0	0
Absence of milk spot	0	0	0	1		0	0
No fur	2	3	16	12	6	15	7
Vocalization	3	1	13	6	9	12	6
Day 14							
Weight	0	0	0	1 ↓	1↑	6↑	3↓
Eyes closed	0	0	21	21	4	9	4
Ears closed	1	0	0	3	9	9	2
No teeth	0	0	0	0	2	3	1
No fur	0	0	0	0	3	11	5
No grip	0	0	0	0	0	1	0
Passive	0	0	0	0	5	3	2
Vocalization	0	0	14	10	0	0	0

*4 mice were found dead but were not genotyped.

Table A2. Number of mouse pups found with development deviations at weaning.

	Nestin PDGF B		**GAD67-GFP**		**Kv1.1**		
Clinical examination	Wild type n=55	Trans-genic n=43	Wild type n=33	Trans-genic n=22	Wild type n=18	Heter ozyg. n=32	Homo zyg. n=13
Dead	0	0	0	1	0	0	0
Body weight	0	0	0	0	1↓ 1↑	6↑	2↓1↑
Body posture	0	0	0	1	2	3	4
Tail posture	0	0	0	0	1	0	2
Gait	0	0	0	0	2	1	1
Teeth/mucosa	0	0	0	0	0	1	0
Skin/ears/paws	0	0	0	0	0	0	1
Body mass	0	0	0	0	2	7	3
Respiration	1	1	0	0	0	0	0
Dehydration	0	0	0	0	2	3	3
Tonus abdomen	0	0	0	0	1	9	1
Tonus fore legs	0	0	0	0	0	0	1
Tonus hind legs	0	0	0	0	0	3	1
Eyes	1	3	1	0	4	10	3
Fur	0	0	0	0	3	5	1
Whiskers	2	6	0	0	0	0	0
Hearing	0	1	0	0	1	2	3
Touch	5	6	5	1	1	0	3
Biting	7	3	32	20	4	8	4
Vertical pole	0	0	0	0	4	3	1
Postural reflex	0	0	0	0	0	2	0
Vocalization	17	6	32	20	9	8	10