Original Article

Laboratory Animals
47(3) 153–161

DOI: 10.1177/0023677213475603
la.sagepub.com
SAGE

Assessment of postsurgical distress and pain in laboratory mice by nest complexity scoring

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Abstract

Preliminary studies have suggested a correlation between postsurgical pain and nest building behaviour in laboratory mice. However, there is no standardized measure for estimating pain by means of nest building performance. Here, we investigated nest building under various conditions, and scored nest complexity to assess postsurgical pain. Mice of both sexes, different strains [C57BL/6J, DBA/2J, and B6D2-Tg(Pr-mSMalphaActin)V5rCLR-25], and kept under different housing conditions, showed no differences in their latency to use the offered nest material. Healthy female C57BL/6J mice were engaged 4.3% of the day with nest building and showed three peaks of this behaviour: in the beginning and middle of the light phase, and in the second half of the dark phase. For assessment of postsurgical pain, female C57BL/6J mice underwent a sham embryo transfer +/− different doses of the analgesic carprofen or control treatment. Nest complexity scoring at 9 h after the experimental treatments (i.e. at the end of the light phase) resulted in less than 10% of animals with noticeably manipulated nest material (nestlet) after surgery and more than 75% of healthy mice having built identifiable-to-complex nests or had noticeably manipulated nestlets, while animals after anaesthesia-only showed intermediate nest complexity. Carprofen analgesia resulted in no (5 mg/kg) or only slight (50 mg/kg) improvement of nest complexity after surgery. Thus, nest complexity scoring can be incorporated into daily laboratory routine and can be used in mice as a sensitive tool for detecting reduced wellbeing and general condition, but probably not for determining the efficacy of pain treatment.

Keywords

Mice, nest building, pain assessment, nest complexity scoring, refinement

The construction of nests is common in rodent species. Wild house mice build nests to provide heat conservation; shelter from elements, predators, and competitors; and to allow successful reproduction.[1–3]

The motivation and ability to perform this complex behavioural sequence culminating in a finished nest persist also in domesticated mice and those in laboratory animal facilities. Aside from 'brood' or maternal nests, built specifically for reproduction, if provided with suitable nest building materials, laboratory mice of both sexes build 'sleeping' or non-maternal nests.[4,5] In the laboratory setting, nests might allow the mouse to shield itself from conspecifics, as well as humans and external stimuli, e.g. direct light.[6] Also, as most animal facilities have ambient temperatures below their thermoneutral temperature, mice might build nests for thermoregulatory reasons.[7,8] The motivation for nest building is high, and nest building material is highly valued by laboratory mice.[9,10]

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Spontaneous, species-specific behaviours, such as nest building, that are performed in the animals' home cage have been proposed in the last decade as useful indicators for welfare assessment in small laboratory animal species, and might be used as simple, non-invasive and reproducible indicators for estimating, e.g. neurological dysfunction or pain. A reduction in these behaviours may signal a change in the motivational state of the animal and good performance seems to be indicative of good wellbeing in animals.[11–15]

Nest building performance has been shown to be sensitive to several diseases and neurological impairments. A decrease in this spontaneous behaviour correlates with brain lesions,[16,17] and genetic mutations,[18] as well as the progression of scrapie and Alzheimer's disease.[19,20] Nest building is compromised by the systemic injection of MPTP, a Parkinson model,[14] and in a model of Rett syndrome.[21] Also, in LPS-treated mice, maternal nest building is decreased significantly.[22]

A previous study from our laboratory revealed a correlation between postsurgical pain and nest building performance in laboratory mice.[12] However, a standardized protocol for the assessment of pain by means of nest building performance has not been developed so far.

In the present study we used 'latency to nest building' and a nest complexity scoring scale similar to already published nest building assessment methods.[1,2,14,23,24]

We analysed the general nest building performance of healthy mice of different strains, sexes and housing conditions to evaluate genetic and environmental influences on nest building.

We assume that successful assessment of nest building performance depends on the right testing time points, as mice tend to destroy and rebuild their nests in a circadian rhythm. Therefore, to determine a suitable testing time we analysed the normal nest building rhythm in healthy C57BL/6J mice.

Using this determined time point, we aimed to prove the feasibility and reliability of nest complexity scoring as a method for detecting mild to moderate postsurgical pain in laboratory mice, and to standardize this method for routine laboratory use. For this purpose, individually-housed female C57BL/6J mice underwent a sham embryo transfer with or without different doses of the analgesic carprofen or underwent control treatment only.

We hypothesized that nest complexity is a sensitive and reliable tool that can be used to assess and grade postsurgical pain in mice.

Materials and methods

Ethics statement

The animal housing and experimental protocols were approved by the Cantonal Veterinary Department, Zurich, Switzerland, under licence no. ZH 120/2008, and were in accordance with Swiss Animal Protection Law. Housing and experimental procedures also conform to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No. 123 Strasbourg 1985) and to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Animals

The animals were 48 female and eight male C57BL/6J, eight female DBA/2J, and eight female B6D2-Tg(Pr-mSMalphaActin)V5rCLR-25 mice, obtained from our in-house breeding facility at the age of 6–8 weeks.

The animals' health status was monitored throughout the experiments by a health surveillance programme according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines. The mice were free of all viral, bacterial, and parasitic pathogens listed in the FELASA recommendations, except for *Helicobacter* species.[25]

All the animals were housed in groups of three to six animals for at least three weeks prior to testing in our animal room. The animals were kept in Eurotype III clear-transparent plastic cages (425 mm × 266 mm × 155 mm) with autoclaved dust-free sawdust bedding and one nestlet™ (5 cm × 5 cm), consisting of cotton fibres (Indulab AG, Gams, Switzerland) as nest building material. They were fed a pelleted and extruded mouse diet (Kliba No. 3436, Provimi Kliba, Kaiseraugst, Switzerland) ad libitum and had unrestricted access to sterilized drinking water. The light/dark cycle in the room consisted of 12/12 h with artificial light (approximately 40 Lux in the cage). The temperature was 21 ± 1°C, with a relative humidity of 45 ± 10%, and with 15 complete changes of filtered air per hour HEPA H13 filter, Camfil AG, Unterägeri, Switzerland. The animal room was insulated to prevent electronic and other noise. Disturbances, e.g. visitors or unrelated experimental procedures in the animal room, were not allowed.

Experiments

Latency to nest building: effects of strain, sex and housing conditions. Latency to first nest building activity was determined in mice of the three different strains, both sexes and under different housing conditions to analyse effects of these factors on nest building performance.

Eight female and eight male C57BL/6J, eight female DBA/2J, and eight female B6D2-Tg(Pr-mSMalphaActin)

V5rCLR-25 mice, housed individually, and eight pairs of female C57BL/6J mice were tested in their familiar home cages. Additionally, eight female C57BL/6J mice, housed individually, were transferred to a new and clean cage directly before testing.

The mice were housed individually or in pairs three days prior to and during the observations. At the start of the 3-day adaptation phase, one nestlet was placed in the cage. Prior to observation at the beginning of the light phase, nestlet material was removed and a new nestlet was placed in the cage at the beginning of the light phase. Animals were video recorded for 24 h with an infrared-sensitive camera fixed above the cage.

All video recordings were analysed with ObserverXT™ 9 software (Noldus, Wageningen, The Netherlands). Nest building activity was defined as manipulating or carrying the nestlet or nestlet material for more than 3 s, and the latency to nest building was recorded in seconds.

Assessment of circadian nest building rhythm. The 24 h observations of eight individually-housed female C57BL/6J mice in their familiar home cage (the same mice as used in the assessment of latency to nest building) were analysed to assess normal circadian nest building rhythms in order to determine the optimal time point for nest complexity scoring.

All video recordings were analysed with ObserverXT™ 9 software, and nest building duration in seconds was recorded continuously. The remaining behaviours were separated into resting (inactive) and other activities and measured in seconds. Additionally nest complexity was scored by carefully approaching the cage without disturbing the animal at eleven time points using the scale described in Figure 1.

Pain assessment with nest complexity scoring. Experimental design: Forty-eight individually-housed female C57BL/6J mice were tested (partly the same mice as used in the previous observations). Animals were tested before (baseline) and after an experimental procedure (experimental). Eight mice were allocated randomly to one of six experimental groups: (1) surgery + anaesthesia (mice underwent anaesthesia and surgery without analgesic treatment), (2) surgery + anaesthesia + low dose analgesia (mice underwent anaesthesia and surgery with 5 mg/kg carprofen), (3) surgery + anaesthesia + high dose analgesia (mice underwent anaesthesia and surgery with 50 mg/kg carprofen), (4) anaesthesia only, (5) anaesthesia + low dose analgesia, (6) anaesthesia + high dose analgesia.

Baseline data acquisition: Mice were housed individually three days prior to and during testing. At the start of the 3-day adaptation phase, one nestlet was placed in the cage. Prior to testing, the nestlet material was removed and a new nestlet was placed in the home cage at the beginning of the light phase.

Nest scoring (Figure 1) was carried out in the animal room by blinded observers 9 h after providing the nestlet, as this was found to be the optimal time point for nest complexity scoring in the analysis of circadian nest building rhythm (see also Results).

Experiments and experimental data acquisition: Experimental scoring of nest complexity was performed 2 days after baseline measurements. The experiment began at 1.5 h before the start of the light phase with a subcutaneous injection of 2 µL/g body weight of phosphate buffered saline (PBS) for the surgery + anaesthesia and anaesthesia-only groups. In the surgery + anaesthesia + analgesia and anaesthesia + analgesia groups, 5 or 50 mg/kg body weight of the analgesic carprofen (Rimadyl™, Pfizer Inc, New York, NY, USA) was diluted in PBS and injected subcutaneously as 2 µL/g body weight. Forty-five minutes later, the animals were transferred in individual transport cages to the operating theatre, which was located nearby. Mice were anaesthetized with sevoflurane (Sevorane™, Abbott, Baar, Switzerland) as a mono-anaesthesia. The anaesthetic gas was provided with a rodent inhalation anaesthesia apparatus (Provet, Lyssach, Switzerland); oxygen was used as a carrier gas. After induction of anaesthesia in a Perspex induction chamber (8% sevoflurane, 600 mL/min gas flow) animals were transferred to a warming mat (Gaymar, TP500, Orchard Park, NY, USA) set at 39 ± 1°C to ensure constant body temperature, and anaesthesia was maintained via a nose mask (6–7% sevoflurane, 600 mL/min gas flow). The fur was clipped and the operating field disinfected with ethanol in all animals. Mice of surgery groups underwent a one-side sham embryo transfer. The incision in the abdominal muscle wall was closed with absorbable sutures (Vicryl™, 6/0 polyglactin 910, Ethicon Ltd, Norderstedt, Germany) and the skin was closed using skin staples (Precise™, 3 M Health Care, St Paul, MN, USA). Surgery was completed within 6–8 min in the surgery groups. Anaesthesia lasted 14–16 min in all groups. Animals were allowed to recover for 15–20 min on the warming mat before being transferred back to the animal room for subsequent behavioural testing.

The testing began at the beginning of the light phase after removing the used nestlet and adding a new nestlet by returning each mouse from its transport cage to its home cage. At 9 h after providing the new nestlet, nest scoring was carried out by carefully approaching the cage without disturbing the animal.

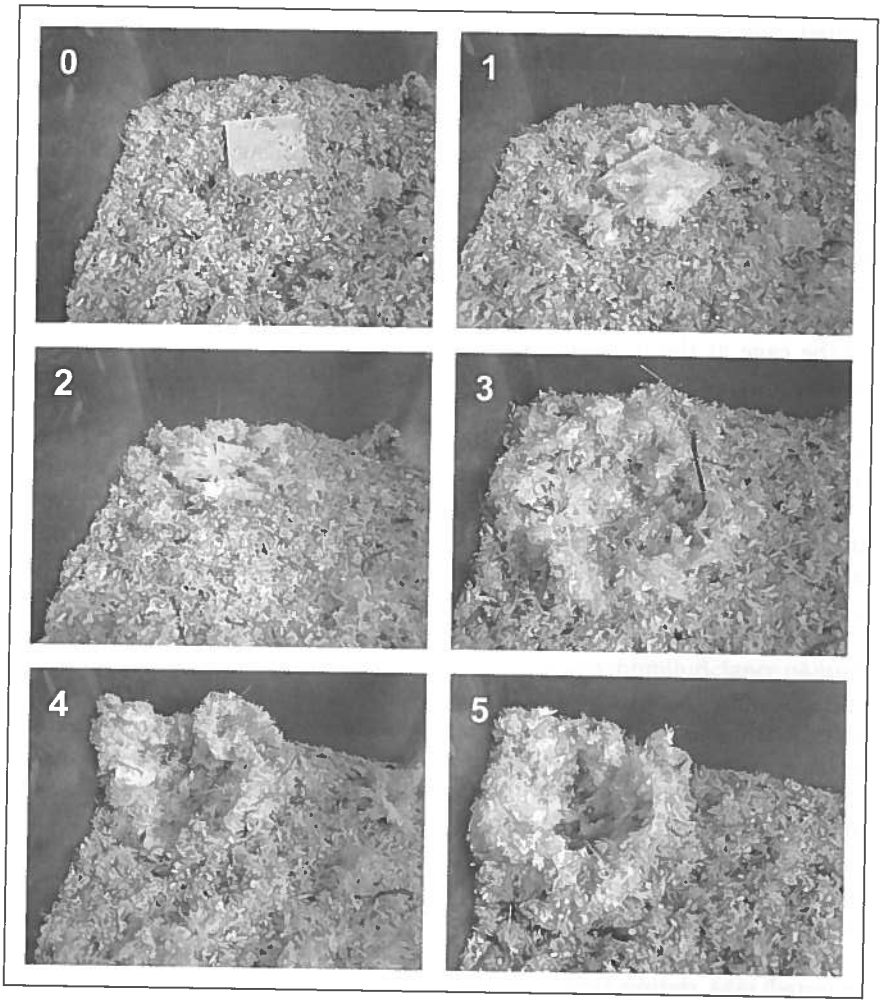

Figure 1. Nest complexity scoring: Score 0 = nestlet not manipulated, possibly dragged around the cage; Score 1 = nestlet slightly manipulated, more than 80% of nestlet intact, possibly a few shreds picked out; Score 2 = nestlet noticeably manipulated, less than 80% of nestlet intact, shreds spread around or in one area; Score 3 = noticeable nest site; less than 80% of nestlet intact, shreds are placed mostly in the nest site, hollow in bedding, mice start building walls; Score 4 = flat nest, hollow in bedding, walls mainly higher than mice and encasing the nest less than 50%; Score 5 = complex nest, more than 50% shreds picked out, bowl-shaped nest, walls higher than mice and encasing the nest by more than 50%.

Statistical data analysis

Statistical analyses were performed using SPSS 20.0 software (IBM, Armonk, NY, USA). All data were tested for normal distribution and homogeneity of variance and met the necessary assumptions for parametric analyses. Mean and standard error of the mean (SEM) of latency to nest building, duration of nest building, and nest complexity scores for baseline and experimental measurements were calculated. Latencies to nest building were compared between different strains or housing conditions with a one-way analysis of variance (ANOVA). To compare the effect of sex on latency to nest building, an independent two-sample t-test was used. To test for significant differences between nest scores general linear model for repeated measures with time as within- and treatment as between-subject factor was used; post hoc testing was conducted with the Bonferroni test. Significance for all statistical tests was established at $P < 0.05$.

Results

Latency to nest building: effects of strain, sex and housing conditions

No significant differences were found in latencies to nest building between strains ($P = 0.415$), sexes ($P = 0.741$), and housing conditions (social environment/physical environment) ($P = 0.871$) (Table 1).

Table 1. Latency to nest building: mean latency (SEM) of all strains, sexes and housing conditions.

Strain	Sex	Housing condition	Mean (min)	SEM (min)
C57BL/6J	Female	Individual housing/familiar cage	60.5	16.5
	Female	Individual housing/new cage	62.5	15.2
	Female	Pair housing/familiar cage	52.5	10.1
	Male	Individual housing/familiar cage	54.6	5.9
DBA/2J	Female	Individual housing/familiar cage	44.3	6.6
B6D2-Tg(PrmSMalphaActin) V5rCLR-25	Female	Individual housing/familiar cage	37.9	11.2

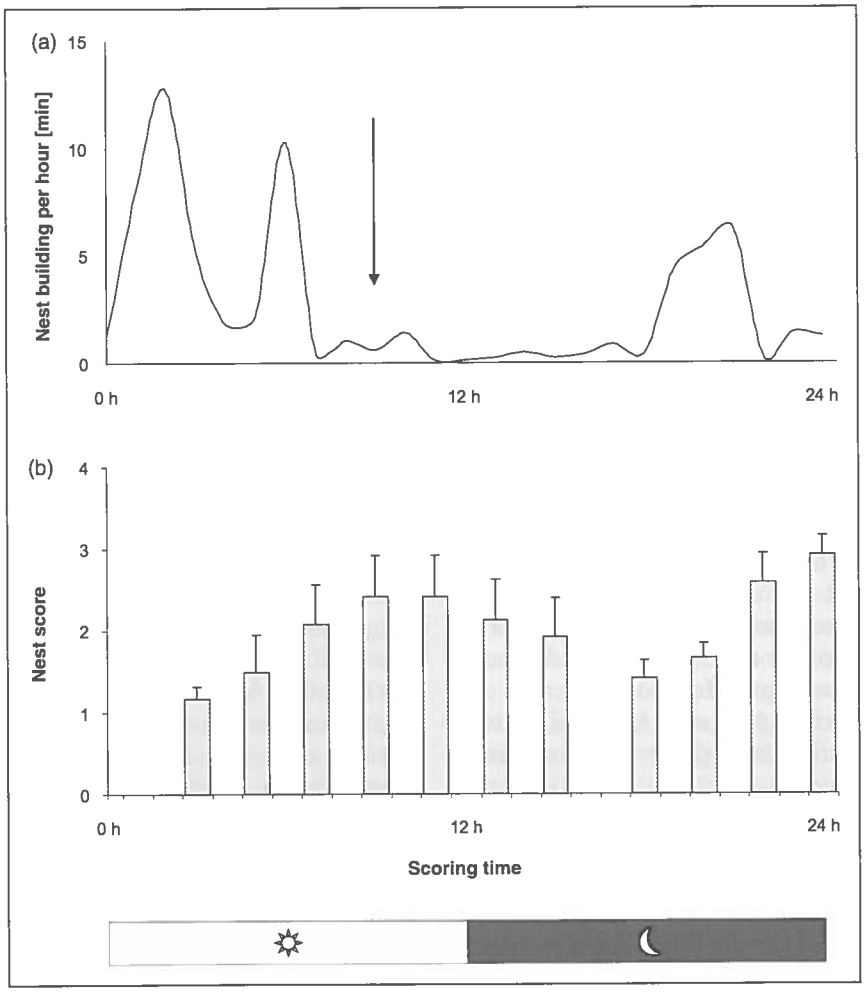

Figure 2. (a) Normal nest building activity during 24 h in female individually-housed C57BL/6J mice. Mean duration of nest building in minutes per observed hour is shown on the vertical axis. (b) Determination of optimal scoring time: Mean nest scores (+SEM) at 11 scoring time points. Scores increased during light phase, decreased at the onset of activity during the dark phase and increased towards a maximum at the end of the dark phase.
Time point of nest complexity scoring for pain assessment is indicated with arrow (9 h after start of the light phase).

Circadian nest building rhythm

Mice were occupied with nest building for, on average, 62.3 (SEM 22.6) min during 24 h of observation, which is equivalent to 4.3% of the day.

On average, mice started with nest building within the first 2 h after nest material was provided. This phase with high nest building activity (Figure 2a) was followed by resting phases with short disruptions for several behavioural activities and short nest building

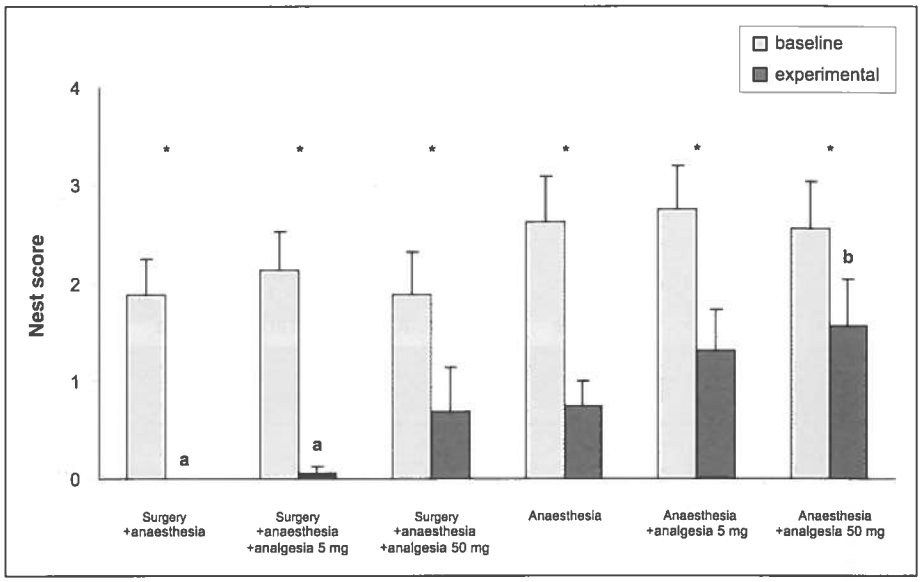

Figure 3. Mean nest complexity scores (+SEM) of female individually-housed C57BL/6J mice at 9 h. Baseline and experimental measurements of all experimental groups are shown. Asterisks indicate within-subject effect of time (baseline versus experimental) at $P < 0.001$. A significant between-subject effect of treatment was found ($P < 0.05$). Different letters over the bars indicate the differences revealed by post hoc analysis between nest scores of experimental groups at $P < 0.05$ (a versus b).

periods for rebuilding or maintaining the nest. In this late light phase nest scores reached high values. During the start of the dark phase, the mice normally had periods of locomotor activity with short breaks for occasional resting. In this active phase, the nest was usually destroyed or flattened out in the bedding by running over the nest site or digging in the bedding, which resulted in a decrease of nest complexity. From the middle to the end of the dark phase, mice started to rebuild their nests, interrupted by resting periods. On average, mice showed higher nest building activity towards the end of the dark phase with increasing nest scores.

Regarding the analysis of the video sequences and the nest scoring, a single time point, 9 h after providing the new nestlet and the start of the light phase, was chosen for determination of the nest complexity scores for pain assessment in the following experiments (Figure 2a and b, arrow).

Nest complexity scoring

Nest scores showed a gradation after experiments (Figure 3). While animals that underwent surgery without or with a low dose of analgesia did not construct noticeable nests, animals treated with higher doses of analgesia had nest scores comparable with anaesthesia-only animals. Animals that received anaesthesia and analgesic treatment only had the highest experimental nest scores.

A significant within-subject effect of time (baseline versus experimental, $P < 0.001$) as well as a significant between-subjects effect of treatments ($P = 0.024$), but no interactions between time and treatment were found. Differences between nest scores of experimental groups were significant when comparing surgery + anaesthesia and surgery + anaesthesia + analgesia 5 mg with anaesthesia + analgesia 50 mg ($P = 0.022$; $P = 0.031$; Figure 3).

Overall, in baseline measurements 75–88% of all mice had noticeable-to-complex nests or at least their cages showed signs of noticeable nestlet manipulation, i.e. scores of two and higher, at the defined scoring time. After the experiments, the percentage of animals with noticeably manipulated nestlets and/or noticeable nests decreased to less than 63%, while animals after surgery without or with a low dose of analgesia never had noticeable nests and only less than 10% of these animals showed noticeable nestlet manipulation (Figure 4).

Discussion

All healthy mice investigated in this study exhibited complex nest building behaviour and constructed nests regardless of strain or sex and under all housing conditions tested. This situation changed distinctly when animals underwent a surgical or anaesthetic procedure: an incremental decrease of nest building performance was observed, correlating with the degree of invasiveness of the experiment.

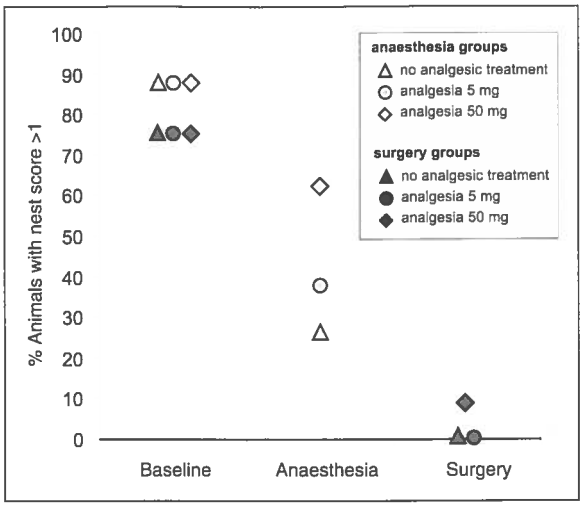

Figure 4. Percentage of female individually housed C57BL/6J mice of different experimental groups with noticeably manipulated nestlet and/or noticeable nests (nest complexity score >1) before and after experimental treatment at 9 hours at the end of the light phase. After experiments (i.) < 10% animals with noticeably manipulated nestlet and/or noticeable nests were found in surgery groups, (ii.) 20 - 60% animals in anaesthesia groups, (iii.) and >75% animals in baseline groups (healthy mice).

We propose that changes in the highly motivated nest building behaviour can be used as a robust indicator of reduced animal wellbeing as also suggested previously by Arras et al.[12] and Deacon[2]. To establish a standardized protocol for the evaluation of postsurgical pain by nest building behaviour, we analysed common factors that could impact on the measurements and consequently influence the transferability of the monitoring protocol. Variability between sexes or genotypes regarding the amount of nest material used (e.g. weight of cotton fibres) or the shape and complexity of the nests have been reported.[4,26] However, in our study, the motivation to use the offered nest building material seemed to be high, and was comparable in different strains, sexes and under the different housing conditions used, as no difference in latency to nest building was observed in healthy mice. Most mice began to manipulate the nest material rapidly and spontaneously within minutes after the material was placed in the cage. Although small inter-individual differences in the shape and complexity of the nests appeared, individuals appeared to be consistent – an observation also described by others.[4]

We then tested the feasibility of nest complexity scoring in a set-up, with female C57BL/6J mice housed individually in their familiar home cages. Since we observed in our daily work that mice in general destroyed their nests during locomotor activity periods, we considered the appropriate choice of testing time to be an important prerequisite for successful complexity scoring. Nest complexity scoring is often done in the morning after material having been provided the day before (see Ref 2 for example), but surgery or other experimental procedures are often conducted during the day, and pain or other impairment are likely to be most significant directly after such procedures. In experiments with minor invasiveness, it is possible that nest scoring 24 h post procedure might miss the most pronounced signs of reduced wellbeing, as nest scores seemed to increase in our study during the dark phase (i.e. at 22 and 24 h after the experiment) up to baseline values (data not shown). Therefore we aimed to identify the appropriate testing time, considering the time point of the experiment and adapting to laboratory routine schedules.

The results of our analyses of behavioural circadian rhythmicity were comparable with the findings of other studies, showing that mice normally build a new nest or repair an old nest at the end of the dark phase.[27,28] Nest building peaks of our mice were shifted towards the beginning of the light phase, which was perhaps associated with the fact that fresh nest building material was provided in this phase. After pronounced nest building activity, animals rested in their nests and nest complexity scores were high and remained relatively constant in this phase. Additionally, this phase with constant nest scores fell within the normal working hours of laboratory personnel, which is a relevant argument for a method that should be applicable under routine laboratory conditions.

Based on these data, we chose a scoring time point of 9 h after experimental treatments at the end of the light phase. However, several hours before this time point also appear to be suitable for successful nest complexity scoring. By this means, nest scoring can act as a short-term retrospective indicator of impairment, which can be applied easily within the normal working day, particularly if procedures are conducted in the early morning. Thus, mice that have suffered, or are still suffering because of inefficient analgesic treatment can be identified easily and can be provided with rescue analgesic treatment.

A total of 75–88% of all healthy mice had identifiable nests or cages that showed at least noticeable nestlet manipulation leading to mean nest scores of 2–3. The maximum scores of 4–5 were difficult to reach within 9 h – in particular for a single-housed mouse – as the nestlet was a quadrate of tightly packed cotton fibres which thus needed intense work to reconstitute into a nest. Therefore we assume that nest scores of two and higher are normal nest scores for healthy individually housed mice after 9 hours.

In contrast to the substantial nest building performance of healthy mice, i.e. nest scores of 2 and above

(see Figure 4), none of the animals undergoing surgery had noticeable nests 9 h after experiments and less than 10% of the animals manipulated the nestlet noticeably, suggesting that these groups may suffer from distress, impaired general condition or even pain.

As observed already in previous studies,[12,13,29] anaesthesia alone had a marked impact on behaviour. Here we observed a distinct effect of anaesthesia on nest building behaviour, which was significantly relative to the baseline, but clearly did not affect the animals as strongly as surgery, indicating only mild impairment. Animals that underwent anaesthesia and additional carprofen treatment of different doses had higher nest scores than anaesthesia-only groups. Whether carprofen can inhibit anaesthesia-induced behavioural aberrations, and thus have a nest building promoting effect, could not be clarified in this study. To our knowledge no behavioural effect of carprofen has been described that might explain these results.

Animals that received the low dose analgesic carprofen before surgery did not show a clear increase in nest complexity, which might be a sign that this dose was too low to relieve postsurgical pain, despite the fact that 5 mg/kg is a standard dose for mice[30] and has been shown to act as an effective analgesic protocol after surgery.[13] In the higher dose (50 mg/kg), we observed a slight tendency towards higher nest complexity comparable with the anaesthesia-only group, although not as high as in anaesthesia with analgesia groups.

As the clear and significant difference in nest complexity between healthy mice and mice that underwent surgery could not be alleviated by carprofen treatment in a significant manner, our study lacked a sound proof that postsurgical pain caused the massive decrease in nest complexity. Thus, other impacts of surgery might also affect nest complexity, e.g. physiological stress or motor impairment. However, it is very unlikely that carprofen in the used dose rates was not capable of pain relief as it has been proved to be effective after laparotomy in several studies (see Refs 13 and 31 for examples). Nest complexity scoring might therefore be a useful indicator of reduced wellbeing after surgery, but cannot be used to assess the efficacy of pain treatment. Nevertheless, the estimation, i.e. grading, of the impact of procedures on wellbeing and general condition is possible with nest complexity scoring.

Our results suggest that deficits of nest building are associated with reduced wellbeing and impaired general condition,[12] which can also include pain, and may trigger a competitive motivational system that makes the animal tend to be lethargic or be concerned with other behaviours like self grooming and with decreased motivation to engage in otherwise highly valued nest building behaviour.[22,32]

Nest complexity scoring is based on the animal's normal behaviour performed in the animal's home cage, and does not require special apparatus or housing facilities. The test causes no additional stress to the animals, as nest building is a species-specific and complex form of active interaction with the environment. Providing nest material allows mice to structure their environment and gain more control over their living conditions, which is assumed to enhance their wellbeing.[10,20,33,34]

In summary, nest complexity scoring can be implemented easily in any laboratory animal facility and can be applied in the daily routine for the detection and assessment of post-procedural impairment in laboratory mice. Even though the motivation to use the nest material was comparable under different conditions, it might be necessary to adapt the scoring system to other nest building material or housing conditions. As social housing is the preferred housing condition for mice and has been suggested to enhance postsurgical recovery in female mice,[15,29,35] further studies should focus on an adaption of the described assessment method to prevalent housing conditions like pair or group housing.

Acknowledgments

This work was sponsored by grants from the Federal Veterinary Office (Bern, Switzerland), and UBS Foundations. The authors would like to thank Robin Schneider and the staff of the Central Biological Laboratory for support in housing the mice. We thank Professor Kurt Bürki for generously providing research facilities and resources.

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Original Article

Laboratory Animals
47(3) 162–167

DOI: 10.1177/0023677213478299
la.sagepub.com
SAGE

Disc1 deletion is present in Swiss-derived inbred mouse strains: implications for transgenic studies of learning and memory

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Abstract

Inbred mouse strains are widely used for genetic studies because of the isogenicity within a strain or F_1 hybrid and the genetic heterogeneity between inbred strains. In the process of modifying *Disc1* in the mouse genome, a 25-bp deletion was discovered in exon 6 of the gene in the 129S6/SvEvTac inbred strain, and subsequently in 16 other inbred strains in the category known as 'Castle's mice'. The deletion ($Disc1^{del}$) induces a frame shift that introduces a premature termination codon, which has been shown to confer an impairment in working memory. To extend knowledge of the distribution of $Disc1^{del}$ among the various inbred strains of laboratory mouse, we investigated whether $Disc1^{del}$ is present in the categories known as 'Swiss mice' and 'strains derived from China and Japan'. We found that the FVB/NJ, SJL/J and SWR/J strains in the 'Swiss mice' category and DDY/JclSidSeyFrkJ in the 'China and Japan' category are homozygous for the $Disc1^{del}$ allele, while ICR/HaJ in the 'Swiss mice' category is homozygous for wild-type *Disc1*. Since the $Disc1^{del}$-positive strains FVB and SJL are commonly used for the generation of transgenic mice, and thus contribute to the genetic background of multiple transgenic lines, our results may allow scientists to avoid the potential confounding effects of the $Disc1^{del}$ allele in transgenic studies of learning and memory.

Keywords

Deletion, disc1, inbred strain, mouse, working memory

DISC1 (*D*isrupted *i*n *sc*hizophrenia *1*) was directly implicated as a candidate gene for psychiatric illness through a balanced translocation t(1;11) that directly disrupts the *DISC1* open reading frame and co-segregates hemizygously with schizophrenia and affective disorder in a large Scottish family.[1,2] Two subsequent resequencing studies found that ultra-rare heterozygous missense mutations in DISC1 are associated with an estimated attributable risk of about 2% in schizophrenia[3] and 0.5% in bipolar disorder,[4] confirming *DISC1* as a promising susceptibility factor for major mental illnesses. The *DISC1* orthologue in mice maps to the mouse syntenic region and shows conserved genomic structure. The mouse *Disc1* and human *DISC1* genes show 60% homology at the DNA level and 56% identity and 14% similarity at the protein level.[5] Although the amino acid sequence of DISC1 is diverging rapidly, several protein motifs are conserved between the orthologues, including leucine zipper, nuclear localization, and coiled-coil domains.[6]

Animal models can be valuable tools for exploring the underlying pathologies of human diseases and developing better therapies. Although it is difficult to model a psychiatric disorder like schizophrenia in animals (e.g. hallucinations and delusions are likely to be human specific), anatomical, behavioural and cognitive characteristics have been described in patients, and it is feasible to study these in animal models.[7] To study the

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consequences of altered DISC1 function on endophenotypes involved in psychiatric illness, efforts have been made to modify *Disc1* in the laboratory mouse genome. In the process of a gene targeting manipulation of *Disc1* in 129S6/SvEvTac-derived embryonic stem (ES) cells, a 25-bp deletion was discovered in exon 6 of the gene.[8] The deletion (Mouse Genome Informatics nomenclature: $Disc1^{del}$) induces a frame shift that introduces a premature termination codon in exon 7.[8] $Disc1^{del}$ does not affect *Disc1* transcript abundance in 129S6/SvEvTac (129S6) mice,[8] but it is unclear what happens at the protein level. Western blotting with an antibody raised against the N-terminal region of Disc1 (amino acids 272–537) detected an ≈100 kDa band in total brain protein extracts from 10-day-old C57BL/6J mice but not from 10-day-old 129S6 mice or 2-month-old mice of either strain,[8] suggesting that the deletion affects an isoform of Disc1 that is not expressed in adulthood. Mouse Disc1 has multiple isoforms at both mRNA and protein levels.[6,9] The absence of an ≈100 kDa protein band in brain tissue from 3-day-old $Disc1^{del}$ homozygotes has been confirmed by Western blotting with a C-terminal antibody (amino acids 131–170).[10] However, a collaborative study using a variety of antibodies generated by independent groups showed almost identical immunoreactivity of Disc1 around 100 kDa (corresponding in size to full-length Disc1) in brain extracts from adult C57BL/6J and 129S6 mice of an unspecified age.[11] After backcrossing of the $Disc1^{del}$ allele onto the C57BL/6J inbred strain, behavioural analysis showed that heterozygotes and homozygotes display a consistent impairment in working memory in a T-maze test,[8] indicating a dominant effect of the deletion.

Inbred mouse strains have long been used for genetic and immunological studies because of the isogenicity within a strain or F_1 hybrid and the genetic heterogeneity between inbred strains. The genealogical chart showing the origins and relationships of inbred strains (ftp://www.informatics.jax.org/pub/datasets/misc/genealogy/genealogy_key.html) assigns each strain to one of seven categories (A–G).[12] Initial screening of several inbred strains showed that $Disc1^{del}$ is absent from the AKR/J, BALB/cJ, C3H/HeJ, CBA/J and DBA/2J strains within the category known as '(B) Castle's mice', which descends from mice used in breeding experiments by William Castle, and from the C57BL/6J strain within the '(E) C57-related mice' category that descends from an original pair of mice bred by Abbie Lathrop.[8,12] Subsequently, we found that $Disc1^{del}$ is absent from C57BL/6NTac within the 'C57-related mice', but is harboured by 16 other inbred strains within 'Castle's mice', including the 101/RI, BTBR T^+ *tf*/J and LP/J strains[13] and 13 other 129 substrains[14] that are all derived from L C Dunn's 'mouse house' at Columbia University.[15] These findings have been confirmed by recent next generation genomic sequencing of 17 inbred strains.[16] Most ES cell lines for gene targeting are derived from the 129 strain, so the potential presence of a genetic lesion known to confer a cognitive impairment in the heterozygous state[8] may have adverse effects on behavioural studies of 129 ES cell-derived gene-targeted mice. Screening of two outbred stocks has revealed that the $Disc1^{del}$ allele is also found within colonies of Slc:ddY and Slc:ICR mice in Japan.[10]

Knowledge of the distribution of $Disc1^{del}$ among the genealogical chart of inbred strains widely used in biomedical research is far from complete, though, as only two of the seven categories of inbred strains have been assayed to date. The objective of this study was thus to establish whether $Disc1^{del}$ is present in representative strains of the categories known as '(A) Swiss mice' that descend from albino mice imported to the USA from Lausanne in Switzerland[17] and '(C) strains derived from colonies from China and Japan'. Here we report that the DDY/JclSidSeyFrkJ, FVB/NJ, SJL/J and SWR/J inbred mouse strains harbour the $Disc1^{del}$ allele.

Materials and methods

DNA samples

DNA samples from individuals of the FVB/NJ (FVB; Stock # 001800), ICR/HaJ (ICR; Stock # 009122), SJL/J (SJL; Stock # 000686) and SWR/J (SWR; Stock # 000689) strains in the 'Swiss mice' category, the DDY/JclSidSeyFrkJ strain (DDY; Stock # 002243) in the 'China and Japan' category, the C57BL/6NJ strain (B6N; Stock # 005304) and the 129P3/J strain (129P3; Stock # 000690) were obtained from the Mouse DNA Resource of the Jackson Laboratory (Bar Harbor, ME, USA). An ear biopsy from an individual of the 129S9/SvEvH (129S9) inbred strain maintained by MRC Harwell (Didcot, UK) was obtained from the animal facility at St James's University Hospital, Leeds, UK. Genomic DNA was isolated from the ear biopsies using a previously described proteinase K method.[18] Following the measurement of DNA concentration by ultraviolet absorbance using a NanoDrop 1000 Spectrophotometer (Labtech International, Lewes, UK), each sample was diluted to 20 ng/μL in ultrapure polymerase chain reaction (PCR) grade water (Bioline Reagents, Cricklewood, UK).

PCR-based genotyping assays

To genotype *Disc1* in the inbred mouse strains, the forward primer (5′-GCT GTG ACC TGA TGG CAC T-3′) and reverse primer (5′-GCA AAG TCA CCG

CAA TAA CCA-3′) that anneal to parts of the *Disc1* gene common to both the deletion and wild-type alleles, flanking exon 6 (the location of the 25-bp deletion), were used in PCRs with template DNA from the 129P3, DDY, FVB, ICR, SJL, SWR, 129S9 and B6N strains, using a previously described method.[14] The B6N and 129P3 strains were used as *Disc1* wild-type and $Disc1^{del}$ controls, respectively. Following thermal cycling and agarose gel electrophoresis, the deletion allele was identified by an amplicon of 171 bp and the wild-type allele by an amplicon of 196 bp.

To verify the presence of the deletion or wild-type allele of *Disc1* in each mouse strain, we used two further PCR-based assays. The deletion specific assay consisted of forward primer (5′-CTG ACC TCT GCC AAC CTG AG-3′) and reverse primer (5′-AGA GGA CAC TGC AGA CAG TTA GG-3′) that were designed to amplify a fragment of 248 bp from the deletion allele of *Disc1* (129P3 strain) but with no amplification from the wild-type allele (B6N strain). Conversely, the wild-type specific assay consisted of forward primer (5′-AGA GCA GGG TTA ATG GCT GA-3′) and reverse primer (5′-GTG GCT CCA CCT GGA AGG-3′) that were designed to amplify a fragment of 266 bp from the wild-type allele of *Disc1* (B6N strain) but with no amplification from the deletion allele (129P3 strain). Reactions having a total volume of 20 µL were carried out inside 0.2 mL eight-well tube strips (Starlab, Newport Pagnell, UK), with each PCR consisting of 7.75 µL ultra-pure PCR grade water (Bioline), 10 µL HotShot Mastermix (containing optimized mixture of *Taq* polymerase, anti-*Taq* polymerase monoclonal antibodies in 2× reaction buffer [6 mmol/L $MgCl_2$] with 400 µmol/L dNTPs and stabilizer) (Cadama Medical, Stourbridge, UK), 0.5 µmol/L each primer (synthesized by Integrated DNA Technologies, Louvain, Belgium), and 20 ng genomic DNA. Reactions were incubated at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 62°C for 45 s (deletion assay) or 63°C for 30 s (wild-type assay), and 72°C for 40 s, followed by 72°C for 10 min in a G-Storm GS482 thermal cycler (Labtech International). Following thermal cycling, each PCR product was mixed with 4 µL blue/orange 6× loading dye (Promega, Southampton, UK) before being loaded next to 0.5 µg 100-bp DNA ladder (Invitrogen, Paisley, UK) onto a 1.5% (w/v) molecular grade agarose gel (Bioline) containing 1× SYBR Safe DNA gel stain (Invitrogen), and electrophoresed in 1× TAE buffer (40 mmol/L Tris-acetate, 2 mmol/L EDTA) at 5 V/cm for 40 min.

DNA sequencing

To corroborate the results of the *Disc1* PCR-based genotyping assays, amplicons encompassing the location of the 25-bp deletion (*Disc1* exon 6) were excised from the gel with a scalpel and purified using a QIAquick gel extraction kit (Qiagen, Crawley, UK). Purified DNA bands at 2 ng/µL from the DDY, FVB, ICR, SJL and SWR strains were submitted to a commercial company (Eurofins MWG Operon, Acton, UK) for Sanger sequencing. Alignment and comparisons of the resulting DNA sequences with the published *Disc1* sequences of the C57BL/6J (wild-type) and 129S6 (deletion) strains[8,14] were performed using Sequencher (Gene Codes, Ann Arbor, MI, USA) and BioEdit Sequence Alignment Editor[19] software.

Results

The PCR assay using primers that anneal to parts of the *Disc1* gene common to both the deletion and wild-type alleles, flanking the location of the 25-bp deletion,[14] amplified fragments of 171 bp from the 129P3 (deletion control), DDY, FVB, SJL, SWR and 129S9 strains, and 196 bp from the B6N (wild-type control) and ICR strains (Figure 1a). Application of the *Disc1* deletion specific PCR primers to the same inbred strains amplified DNA fragments of 248 bp from template DNA from the 129P3 (deletion control), 129S9, DDY, FVB, SJL and SWR strains, but showed no amplification from the B6N (wild-type control) and ICR strains (Figure 1b). In parallel, the *Disc1* wild-type specific PCR primers amplified DNA fragments of 266 bp from template DNA from the B6N (wild-type control) and ICR strains, but showed no amplification from the 129P3 (deletion control), 129S9, DDY, FVB, SJL and SWR strains (Figure 1b). Sequencing of amplicons encompassing *Disc1* exon 6, followed by alignment with the published *Disc1* sequences of the deletion and wild-type alleles,[8,14,16] confirmed that FVB, SJL and SWR in the 'Swiss mice' category and DDY in the 'China and Japan' category are homozygous for the $Disc1^{del}$ allele, while ICR in the 'Swiss mice' category is homozygous for wild-type *Disc1* (Figure 2).

Discussion

The objective of this study was to establish whether the $Disc1^{del}$ allele is present in representative strains of the categories known as '(A) Swiss mice' and '(C) strains derived from colonies from China and Japan'. Using three independent PCR-based genotyping assays, we found that FVB, SJL and SWR in the 'Swiss mice' category and DDY in the 'China and Japan' category are homozygous for the $Disc1^{del}$ allele, while ICR in the 'Swiss mice' category is homozygous for wild-type *Disc1*. These findings were confirmed by sequencing of PCR amplicons that encompass the location of the 25-bp deletion (exon 6) in the *Disc1* gene. Since inbred

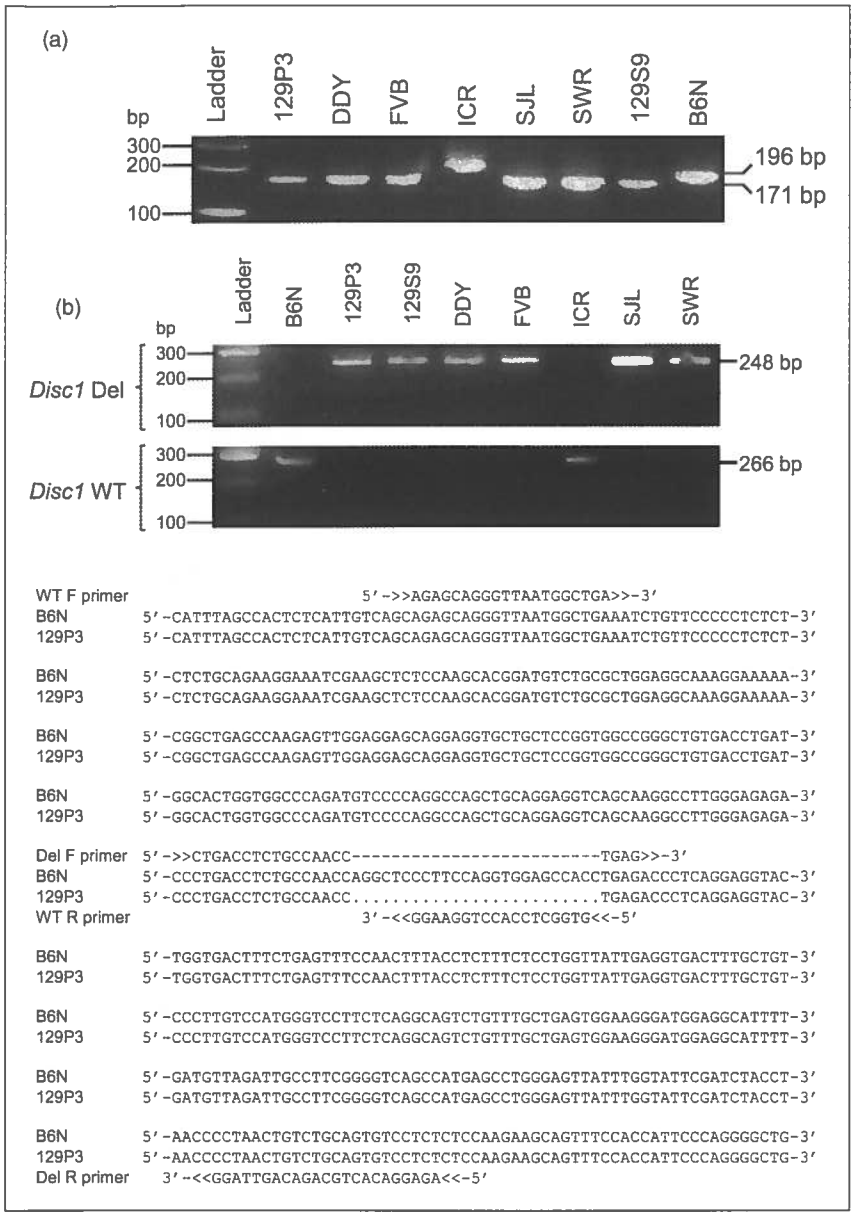

Figure 1. *Disc1* deletion genotyping of inbred mouse strains. (a) Polymerase chain reaction (PCR) assay using primers common to both the deletion and wild-type alleles of *Disc1* amplifies fragments of 171 bp from the deletion (129P3) and 196 bp from the wild-type (B6N) allele. The sizes of the DNA ladder fragments and PCR products are indicated on the sides of the figure. Ladder, 0.5 μg 100-bp DNA ladder (100 bp, 48 ng; 200 bp, 25 ng; 300 bp, 29 ng); 129P3, 129P3/J (deletion control); DDY, DDY/JclSidSeyFrkJ; FVB, FVB/NJ; ICR, ICR/HaJ; SJL, SJL/J; SWR, SWR/J; 129S9, 129S9/SvEvH; B6N, C57BL/6NJ (wild-type control). (b) PCR assays specific for the deletion or wild-type allele of *Disc1*. The B6N strain has a wild-type *Disc1* allele, while the 129P3 strain has a 25-bp deletion (dots) in *Disc1*. The wild-type specific primers (F: 5′-AGA GCA GGG TTA ATG GCT GA-3′; R: 5′-GTG GCT CCA CCT GGA AGG-3′) amplify a fragment of 266 bp from the wild-type allele of *Disc1* (B6N) but not from the deletion allele (129P3). The deletion specific primers (F: 5′-CTG ACC TCT GCC AAC CTG AG-3′; R: 5′-AGA GGA CAC TGC AGA CAG TTA GG-3′) amplify a fragment of 248 bp from the deletion allele of *Disc1* (129P3) but not from the wild-type allele (B6N).

strains are homozygous at every locus, and the alleles at each locus are identical by descent, we conclude that $Disc1^{del}$ is fixed in the FVB, SJL and SWR inbred strains, while wild-type *Disc1* is fixed in the ICR inbred strain. We also found that the $Disc1^{del}$ allele is present in the 129S9 inbred strain, confirming fixation of $Disc1^{del}$ allele throughout the 129 family.

Our finding that the $Disc1^{del}$ allele is present in the categories of inbred strains known as '(A) Swiss mice' and '(C) strains derived from colonies from China and

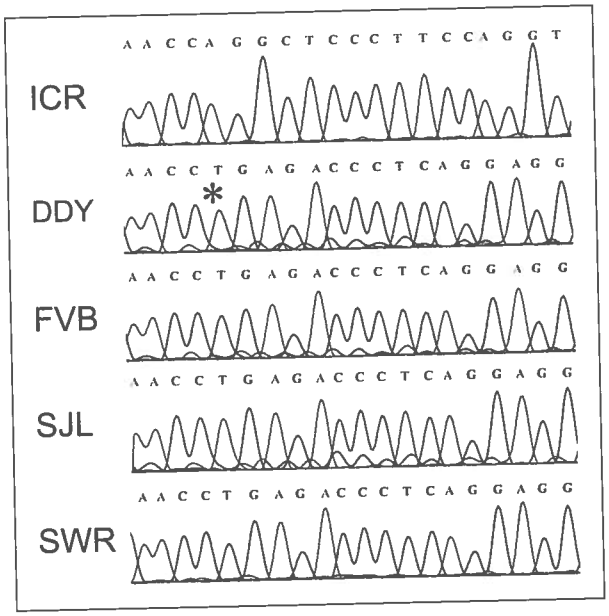

Figure 2. Sequence confirmation of *Disc1* deletion in inbred strains. The sequence chromatograms show the deletion in DDY, FVB, SJL and SWR, and the wild-type allele of *Disc1* in ICR. An asterisk indicates the location of the deletion in the DDY sequence.

Japan', as well as in the category known as '(B) Castle's mice',[8,14,16] indicates that the *Disc1*^{del} allele is more widespread than was previously thought. Although the genealogical chart of inbred strains does not indicate any links between the DDY strain, the inbred Swiss strains and Castle's mice,[12] it is reasonable to postulate that the 25-bp deletion in the *Disc1* gene arose in progenitor stock that contributed to the genetic make-up of these inbred strains as well as two commercially available outbred stocks in Japan (Slc:ddY and Slc:ICR).[10] The DDY inbred strain and the Slc:ddY outbred stock both descend from the same non-inbred 'dd' stock at the University of Tokyo.[12,20] This shared ancestry is consistent with the *Disc1*^{del} allele being present in each of them. The FVB, SJL and SWR inbred strains and the Slc:ICR outbred stock all descend from two male and seven female albino mice derived from a non-inbred stock in the laboratory of Dr A de Coulon at the Centre Anticancéreux Romand, in Lausanne, Switzerland. These animals were imported into the USA by Dr Clara Lynch of the Rockefeller Institute in 1926.[12,21,22]

The finding that the ICR inbred strain is *Disc1* wild-type was unexpected in light of the other inbred Swiss strains tested (FVB, SJL and SWR) being *Disc1*^{del} homozygous. The inbred ICR strain and the Slc:ICR outbred stock both descend from the same Ha/ICR progenitor stock established at the Institute of Cancer Research in 1948.[12,21,22] Since 22.5% of Slc:ICR mice tested were found to harbour the wild-type allele of *Disc1*[10], it is reasonable to postulate that this allele was present in the common progenitor stock and became fixed in the inbred ICR strain during its development. Of the *Disc1*^{del}-positive inbred Swiss strains, FVB and SJL are of particular importance as they are commonly used for the generation of transgenic mice, and thus contribute to the genetic background of multiple transgenic lines,[23] such as a mouse model of Alzheimer's disease with an F_1 hybrid C57BL/6J × SJL/J genetic background[24] and a model of Huntington's disease with an FVB genetic background.[25] To avoid potential confounds resulting from the *Disc1*^{del} allele, scientists using the FVB or SJL strains to generate transgenic mice for studies of brain function would be advised to genotype their transgenic lines for the *Disc1* deletion.

Acknowledgements

This work was supported by a Medical Research Council grant (G0900625) and a Young Investigator Award from the National Alliance for Research on Schizophrenia and Depression to SJC.

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