A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse

Patrick M. Nolan[1], Jo Peters[1], Mark Strivens[1], Derek Rogers[2], Jim Hagan[2], Nigel Spurr[3], Ian C. Gray[3], Lucie Vizor[1], Debra Brooker[1], Elaine Whitehill[1], Rebecca Washbourne[1], Tertius Hough[1], Simon Greenaway[1], Mazda Hewitt[1], Xinhong Liu[1], Stefan McCormack[1], Karen Pickford[1], Rachael Selley[1], Christine Wells[1], Zuzanna Tymowska-Lalanne[1], Phil Roby[1], Peter Glenister[1], Claire Thornton[1], Caroline Thaung[1,9], Julie-Anne Stevenson[1], Ruth Arkell[1], Philomena Mburu[1], Rachel Hardisty[1], Amy Kiernan[4], Alexandra Erven[4], Karen P. Steel[4], Stephanie Voegeling[5], Jean-Louis Guenet[5], Carole Nickols[6], Ramin Sadri[6], Mahmood Naase[6], Adrian Isaacs[7], Kay Davies[7], Mick Browne[3], Elizabeth M.C. Fisher[8], Jo Martin[6], Sohaila Rastan[3], Steve D.M. Brown[1] & Jackie Hunter[2]

As the human genome project approaches completion, the challenge for mammalian geneticists is to develop approaches for the systematic determination of mammalian gene function. Mouse mutagenesis will be a key element of studies of gene function[1–3]. Phenotype-driven approaches using the chemical mutagen ethylnitrosourea[4–6] (ENU) represent a potentially efficient route for the generation of large numbers of mutant mice that can be screened for novel phenotypes. The advantage of this approach is that, in assessing gene function, no *a priori* assumptions are made about the genes involved in any pathway. Phenotype-driven mutagenesis is thus an effective method for the identification of novel genes and pathways[1,2]. We have undertaken a genome-wide, phenotype-driven screen for dominant mutations in the mouse. We generated and screened over 26,000 mice, and recovered some 500 new mouse mutants. Our work, along with the programme reported in the accompanying paper[7], has led to a substantial increase in the mouse mutant resource and represents a first step towards systematic studies of gene function in mammalian genetics.

We treated male BALB/c mice with ENU, mated them with C3H females and screened F1 hybrid progeny (using a variety of protocols) for new mutant phenotypes. We obtained 26,047 F1 progeny and established a control cohort of F1 mice from non-mutagenized BALB/c males to set baseline parameters. We screened all mice at birth and weaning for visible anomalies such as craniofacial, limb and tail defects (Table 1 and Fig. 1). At five weeks, we screened mice using the SHIRPA (for SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment) protocol[8]. SHIRPA is a comprehensive phenotype assessment tool, involving a battery of up to 40 simple tests, that is used to detect mutants in areas such as lower motor neuron/muscle function, spinocerebellar function, sensory function, neuropsychiatric function and autonomic function. We screened 13,375 F1 mice using SHIRPA. A large fraction of progeny (Tables 1 and 2) has also undergone two behavioural testing protocols at six weeks of age. First, we used measurements of locomotor activity (LMA) to identify deficits in motor function. Abnormally high or low activity may be indicative of neurobehavioural deficits. Second, we undertook screens for abnormal acoustic startle response and deficits in pre-pulse inhibition (PPI) of the acoustic startle response. Deficits in PPI have been associated with a number of psychiatric disorders in human[9]. We screened 8,500 F1 mice for abnormal LMA and 9,000 mice for deficits in PPI. We also instituted clinical chemistry screens and analysed blood from 1,454 F1 mice for 17 parameters (Table 1). All data from the mutagenesis programme, including phenotypes, are archived on Mutabase, a

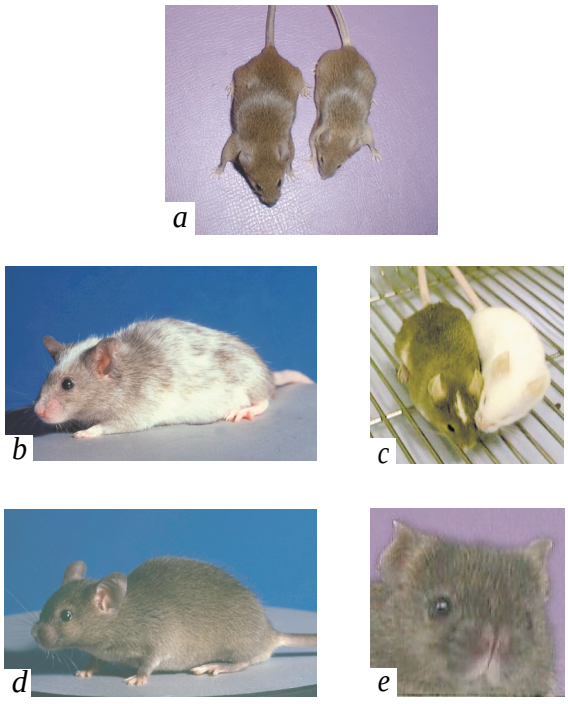

Fig. 1 A few mutants with visible phenotypes detected in the mutagenesis programme. ***a***, Nanomouse (*Nano*), GENA50. ***b***, Dominant spotting ($Kit^{W\text{-}39H}$), GENA133. ***c***, A microphthalmia mutant, GENA163. ***d***,***e***, Batface, a craniofacial mutant, GENA123.

[1]*MRC Mammalian Genetics Unit and Mouse Genome Centre, Harwell, UK. Departments of* [2]*Neuroscience and* [3]*Biotechnology & Genetics, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Harlow, UK.* [4]*MRC Institute of Hearing Research, University Park, Nottingham, UK.* [5]*Institut Pasteur, Paris, France.* [6]*Department of Morbid Anatomy, Queen Mary and Westfield College, London, UK.* [7]*Department of Human Anatomy and Genetics, University of Oxford, Oxford, UK.* [8]*Department of Neurogenetics, Imperial College, London, UK.* [9]*MRC Human Genetics Unit, Edinburgh, UK. Correspondence should be addressed to S.D.M.B. (e-mail: s.brown@har.mrc.ac.uk).*

Table 1 • Screening protocols

Screening for visible defects	Birth	Pre-weaning	Weaning	5 weeks	6 weeks	8–12 weeks
Category				SHIRPA	Behavioural testing	Clinical chemistry
				semi-quantitative	LMA:	
size	large/small	large/small	large/small	battery of tests*	activity recorded for	sodium
sense organs	eye size/colour		eye size/colour	deficits recorded in:	35 min. in cages equipped	potassium
	low set ears		ear size/position	lower motor	with beam-splitting devices	chloride
skin and hair	anaemia	stripes	coat colour/texture	neuron/muscle function		creatinine
	skin colour/texture	skin colour	loose/tight skin	spinocerebellar function	Acoustic startle response	urea
		blotchy coat	greasy/rough coat	sensory function	and PPI of the	total calcium
	curly whiskers		curly coat/whiskers	neuropsychiatric function	acoustic startle response	inorganic phosphate
			thin/balding coat	autonomic function	40 min test in soundproofed	glucose
			dark footpads		startle chambers	bicarbonate
behaviour	activity	activity	activity		incorporating both startle	alkaline phosphatase
		tremors/fits	tremors/fits		and PPI sessions	alanine
		circling	circling			aminotransferase
		head weaving	head weaving			asparate
		ataxia/gait	ataxia/gait			aminotransferase
skeleton	micrognathia		micrognathia			total protein
	agnathia		short/wide/thin head			albumin
	short head					total cholesterol
	scoliosis		scoliosis			HDL cholesterol
	hare lip					triglycerides
tail/extremities	short/bent tail		short/bent tail			
	poly/syndactyly		poly/syndactyly			
	fused toes		fused toes			
	limbs					
	bent/short limbs		bent/short limbs			
	puffy limbs/tail		puffy limbs/tail			
colour/spotting		belly spot	belly spot			
		head blaze	head blaze			
		coat colour	coat colour			
various	blebs/bruising					
	oedema	hydrocephaly	hydrocephaly			
	hydrocephaly					
	chylous ascites					
	spina bifida					

*See http://www.mgu.har.mrc.ac.uk/mutabase.

distributed data recording system specifically developed for this programme (http://www.mgu.har.mrc.ac.uk/mutabase).

From the various screens, we identified 1,089 mice with visible or other anomalies. We introduced 339 mice into inheritance testing by mating founder F1s with C3H (each potential mutant being assigned a GENA number). Routinely, we recovered and rescreened 20–25 progeny from these matings to assess inheritance of dominant phenotypes. We confirmed 126 phenotypes as inherited (Table 2) and 140 phenotypes as not inherited (which includes mice that failed to breed due to illness or fertility problems and those that died before inheritance testing). Also, 73 potential mutants are still in inheritance testing. Thus approximately 50% of the phenotypes uncovered in the programme are true inherited dominant mutations. Overall, we can extrapolate that we have recovered approximately 500 mutants from the screening programme. The rate of recovery of dominant mutations is approximately 2%. We have successfully recovered mutants in all phenotypic areas screened (Table 2). Finally, during inheritance testing we observed only one instance of segregation of elements of the mutant phenotype, GENA191. Whereas the founder mutant mouse showed both abnormal gait and corneal opacity, these two phenotypes appeared to segregate in the progeny. This indicates that, despite the fact that ENU would be expected to introduce multiple hits around the genome, most mutant phenotypes are effectively monogenic.

We used fresh or frozen spermatazoa from original mutant F1 founders (or alternatively from male backcross progeny of founders) to undertake speed backcrosses using *in vitro* fertilization[10] (IVF), mapping a significant fraction of the 126 confirmed mutants. Sperm from mutant animals was used to fertilize C3H oocytes for the rapid, simultaneous generation of large numbers of backcross progeny. Normally, over 100 backcross progeny were produced from each IVF session, scored and tail tipped for DNAs. We used a rapid genotyping approach involving pooling of affected DNAs and fluorescent genotyping of DNA pools using a set of over 200 SSLPs spanning the mouse genome. Overall, we mapped 30 of the confirmed mutants, allowing us to establish that many carry mutations at previously unidentified loci in the mouse genome. In total, 13 mutants (GENA22, 29, 38, 42, 50, 65, 70, 77, 78, 104, 161, 175 and 180) appear to represent novel mutations, of which 3 (22, 50 and 161) appear to be X-linked on the basis of male lethality. We confirmed eight mutations with phenotypes suggesting they represented alleles of known loci on the basis of allelism tests. We mapped six circling mutations (25, 41, 47, 52, 60 and 137), indicative of a vestibular defect, to proximal chromosome 4 in the region of the wheels (*Whl*) allele[11] that may represent new alleles at this locus (manuscript in preparation). Finally, two mutations (51 and 57) were mapped to chromosome 11 and identified as mutations at the *Pmp22* locus (manuscript submitted). Thus, this mutagenesis programme has extended both the breadth and the depth of the mouse mutant resource.

We have undertaken the archiving of novel phenotypes arising from the mutagenesis programme irrespective of their status as confirmed inherited mutations. All male F1 progeny demonstrating detectable phenotypes are archived by sperm freezing[10]. In addition, ovaries from females with abnormal phenotypes are frozen. All phenotypic data can be perused at the ENU Mutagenesis Programme web site (http://www.mgu.har.mrc.ac.uk/mutabase/). All mutants are freely available to non-commercial researchers without restrictions on research and can be requested at the above web site.

Table 2 • Numbers of mice tested along with phenotypes and inherited mutations identified

Weaned and scored for visible phenotype	26,047
SHIRPA tested	13,375
LMA testing	8,500
PPI testing	9,000
Clinical chemistry	1,454
Abnormal phenotypes identified	1,089
Phenotypes introduced to inheritance testing	339
Inherited mutations	126
Not inherited (including lines lost to illness/sterility)	140
Lines currently in inheritance testing	73
Phenotype classes for inherited mutations	
colour and white spotting	19
skin and hair texture	8
size	17
craniofacial	11
tail	4
clinical chemistry	4
internal defect – chylous ascites	1
circling and deaf	16
eye defects	10
neurological and neuromuscular	12
behavioural	22
renal	2

The distribution of inherited mutations among various phenotypic classes is also shown.

Methods

Animals. We carried out animal studies under the guidance issued by the Medical Research Council[12] and Home Office Project Licence no. 30/1517. For this study, we used male BALB/c mice (Charles River). To determine the most appropriate dosage regimen for BALB/c males, we injected males intraperitoneally at ~10 weeks of age with 2×80, 2×100, 1×160 or 1×200 mg/kg ENU (Sigma). We recorded average length of temporary sterility, lethality of injected males, average litter size and average number of dominant phenotypes per injected male. Procedures for ENU administration, including detailed safety procedures, have been described[13]. Comparison of pilot data indicated that the 2×100 mg/kg dose was the most effective for BALB/c males. We injected batches of 300 BALB/c males at 6 monthly intervals to ensure a constant weekly supply of up to 300 F1 progeny for phenotypic screens. A maximum of 50 progeny was screened per BALB/c male injected. Phenotypic screens were routinely carried out on F1 progeny of mutagenized males mice crossed to C3H/HeN females (Charles River), as well as on mouse lines selected for inheritance testing, on inbred strains and on a control cohort of BALB/c×C3H/He F1 hybrid mice. Mating cages contained one male and up to two females. Following weaning at three weeks of age, the screening population was group housed in cages of up to five mice. For inheritance testing, we generally backcrossed F1 mice to the C3H/He strain and classified at least 20 progeny for the phenotype identified in the founder.

Dysmorphology screen. We classified progeny of mutagenized males at birth and up to weaning. Size defects at birth were recorded by weighing the abnormal neonate plus two siblings of the same sex.

SHIRPA screen. At five weeks of age, we assessed all mice using the SHIRPA protocol[8]. The screen, involving a battery of up to 40 simple tests, is semi-quantitative and based on modifications to earlier screens[14]. The procedure was carried out in a simple testing arena and took ~10 min per mouse. Once abnormal phenotypes at SHIRPA were identified, an independent operator confirmed results. Full details on setting up and effecting the SHIRPA screen are available (http://www.mgc.har.mrc.ac.uk/mutabase/shirpa_summary.html).

Quantitative behavioural screens. For the purposes of this programme, we carried out two simple behavioural screens on F1 progeny at six weeks of age. To monitor locomotor activity, we analysed mice in cages equipped with beam-splitting devices (Benwick Electronics). Activity was measured by recording the number of beam splits and the number of cage transitions over a 35-min test period in bins of 5-min duration. Total activity

Table 3 • Map positions of mutations from the mutagenesis programme

Loci mapped by backcrosses[a]			
(GENA number, name, symbol)	Phenotype	Map position	Interval
25 tornado, *Todo*	circling	prox chr 4	Cen (0 cM) – *D4Mit181* (2.5 cM)
29 kumba, *Ku*	belly spot and curly tail	chr 14	*D14Mit137* (6.5 cM) – *D14Mit239* (42.5 cM)
38 sickly, *Sic*	small	distal chr 7	*D7Mit253* (41.5 cM) – Tel (67.8 cM)
41 orbitor, *Obt*	circling	prox chr 4	Cen (0 cM) – *D4Mit181* (2.5 cM)
42 whitetoes, *Whto*	belly spot, white hind toes	prox chr 7	Cen (0 cM) – *D7Mit229* (18.6 cM)
47 dizzy, *Dz*	circling	prox chr 4	Cen (0 cM) – *D4Mit181* (2.5 cM)
51 trembler-1H, *Pmp22^{Tr-1H}*	demyelinating	mid chr 11	*D11Mit131* (25.1 cM) - *D11Mit177* (35.0 cM)
52 cyclone, *Cyn*	circling	prox chr 4	Cen (0 cM) – *D4Mit181* (2.5 cM)
57 trembler-2H, *Pmp22^{Tr-2H}*	demyelinating	mid chr 11	*D11Mit131* (25.1 cM) - *D11Mit177* (35.0 cM)
60 eddy, *Edy*	circling	prox chr 4	Cen (0 cM) – *D4Mit181* (2. 5cM)
65 van gogh, *Vng*	craniofacial, lowered ear	mid chr 5	*D5Mit10* (54 cM) - *D5Mit161* (70 cM)
70 blind drunk, *Bdr*	neurological	mid chr 2	*D2Mit106* (61.2 cM) – *D2Mit311* (83.1 cM)
77 dark footpads 2, *Dfp2*	dark footpads	prox chr 4	Cen (0 cM) – *D4Mit214* (17.5 cM)
78 saggy, *Sagg*	loose skin	prox chr 1	*D1Mit232* (20.8 cM) - *D1Mit234* (25.7 cM)
104 jeff, *Jf*	deaf	distal chr 17	*D17Mit72* (47.4 cM) - *D17Mit123* (56.7 cM)
137 ferris, *Fer*	circling	prox chr 4	Cen (0 cM) – *D4Mit214* (17.5 cM)
175 spin cycle, *Scy*	head bobbing, fits	mid chr 15	*D15Mit60* (17.5 cM) – *D15Mit43* (63.4 cM)
180 robotic, *Rob*	robotic motion	mid chr 5	*D5Mit308* (30.6 cM) – *D5Mit177* (45.9 cM)
Loci mapped by allelism[b]			
(GENA number, symbol)	Mutant name	Map position	Allele names
27, 39, 49, 124 *Kitl*	steel	chr 10	*Kitl^{Sl-35H}*, *Kitl^{Sl-36H}*, *Kitl^{Sl-37H}*, *Kitl^{Sl-38H}*
101 *Eda*	tabby	chr X	*Eda^{Ta-40H}*
108 *T*	brachyury	chr 17	*T^{39-H}*
133, 164 *Kit*	dominant spotting	chr 5	*Kit^{W-39H}*, *Kit^{W-40H}*
X-linked loci[c]			
(GENA number, name, symbol)	Phenotype		Allele names
6 bare patches, *Nsdhl*	bare patches	new allele at *Nsdhl*	*Nsdhl^{Bpa-8H}*
22 dominant sex linked anaemia, *Heph^{sla}*	anaemia		
50 nanomouse, *Nano*	small and pointed head		
161 striped greasy, *Strg*	striped and greasy coat		

[a]The bulk of mutations was localized using speed backcrosses. [b]A number of mutations were mapped using allelism tests. [c]Four mutations demonstrated X-linked inheritance, three of which were novel loci.

and transitions were measured over the last 30 min of this time period. Values in the first 5-min bin were ignored due to extreme variations in results. To monitor acoustic startle response and pre-pulse inhibition of the acoustic startle response (PPI), we tested mice in soundproofed custom-built startle chambers. Tests were divided into startle and PPI sessions and the entire procedure lasted ~40 min. For the startle response, a session consisted of a 5-min acclimation period followed by three types of trials presented a total of 15 times each in pseudorandom order: 110-db pulse at a frequency of 12 or 20 kHz, or white noise. Responses were expressed in arbitrary units and averaged for each type of trial. Duration of pulses was 10 ms and the interval between pulses was 25 s. For PPI, animals received either a pulse alone (white noise, 110 db) or pulse preceded by a 90-db pre-pulse at a frequency of either 12 or 20 kHz. The interval between pre-pulse and pulse was 100 ms. Again each trial was presented a total of 15 times in pseudorandom order. Startle responses of mice when given a pulse following a pre-pulse were expressed as a percentage of the pulse response alone and values were averaged over the trial period. For LMA, startle and PPI testing, mice with any measured value greater than 3 s.d. from the population mean were considered as outliers. The behaviour of an individual was confirmed by retesting after one week.

Clinical biochemistry. We carried out blood sampling between 8 and 12 weeks of age. Plasma (125 µl) was required to perform the current standard profile of 17 clinical assays using an Olympus AU400 analyser. Two criteria were used to identify potential outliers: mice with values greater than 3 s.d. from the mean for any one parameter or greater than 2 s.d. from the mean for groups of related parameters. We retested mice meeting either of these criteria after one month.

Cryopreservation of mouse spermatozoa and mouse ovarian tissue and IVF of mouse oocytes. Sperm freezing and the use of IVF have been described[10]. For ovary freezing, we cut ovaries from mature mice into several pieces and froze them in a cryoprotectant solution of dimethyl sulphoxide (1.5 M) and 10% fetal bovine serum in medium M2 as described[14]. The pieces of ovary were placed in cryoprotectant (0.3 ml) in 1.8-ml Nunc cryotubes and held on ice for 20 min. The samples were cooled to –8 ºC at 2 ºC/min where ice formation (seeding) was induced by touching the cryotubes near the meniscus of the cryoprotectant with forceps cooled in liquid nitrogen. Cooling was then continued at 0.3 ºC/min to –40 ºC, whereupon the samples were plunged into liquid nitrogen and stored in liquid nitrogen.

Genetic mapping. We took tail snips from backcross mice and extracted DNA using a Nucleon HT extraction kit. For each mutant line, we combined equimolar aliquots of DNA from mutant mice and scanned duplicate DNA pools with a set of up to 229 CA repeat markers spanning the mouse genome with a spacing of <20 cM. Markers were derived from the Whitehead/MIT database (http://www-genome.wi.mit.edu/). For those strains with a fully penetrant mutant phenotype, genotyping of the mutant DNA pools was complemented by analysis of pools derived from non-mutant backcross animals. We used a minimum of 40 mice for each genome scan. Putative hits from the pooled scan were confirmed by genotyping individual mice. We mapped five circling mutants, 25, 41, 47, 52 and 60, using agarose gel electrophoresis, and details of procedures for the mapping of these mutants will be reported elsewhere (manuscript in preparation).

Data capture and analysis. All raw and derived data from the programme were stored on a custom designed, distributed database system, Mutabase (http://www.mgu.har.mrc.ac.uk/mgu-about/infor.html). This system comprises an industrial strength database (Sybase Adaptive Enterprise Server, Sybase), application middleware (including World-Wide Web, CORBA and JDBC interfaces) and a standard World-Wide Web browser, client interface. The user interface for Mutabase gives access to functions related to animal husbandry and tracking, behavioural and other screens, and a number of tools to analyse and summarize data. Mutabase is accessible through generic workstations (Windows NT) running a World-Wide Web browser (Netscape Communicator, Netscape) connected to servers via a 100 Mbit network. We used customized barcode technology for both cage and sample labelling, reducing transcription errors when entering data into Mutabase.

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1. Peters, J. & Brown, S.D.M. Combining mutagenesis and genomics in the mouse—closing the phenotype gap. *Trends Genet.* **12**, 433–435 (1996).
2. Brown, S.D.M. & Nolan, P.M. Mouse mutagenesis—systematic studies of mammalian gene function. *Hum. Mol. Genet.* **7**, 1627–1633 (1998).
3. Justice, M., Noveroske, J.K., Weber, J.S., Zheng, B. & Bradley, A. Mouse ENU mutagenesis. *Hum. Mol. Genet.* **8**, 1955–1963 (1999).
4. Russell, W.L. *et al.* Specific locus test shows ethylnitrosourea to be the most potent mutagen in the mouse. *Proc. Natl Acad. Sci. USA* **76**, 5918–5922 (1979).
5. Shedlovsky, A, McDonald, J.D., Symula, D. & Dove, W.F. Mouse models of human phenylketonuria. *Genetics* **134**, 1205–1210 (1993).
6. Rinchik, E.M. & Carpenter, D.A. *N*-ethyl-*N*-nitrosourea mutagenesis of a 6- to 11-cM subregion of the *Fah-Hbb* interval of mouse chromosome 7: completed testing of 4,557 gametes and deletion mapping and complementation analysis of 31 mutations. *Genetics* **152**, 373–383 (1999).
7. Hrabe de Angelis, M. *et al.* Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nature Genet.* **25**, 444–447 (2000).
8. Rogers, D.C. *et al.* Behavioural and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm. Genome* **8**, 711–713 (1997).
9. Geyer, M.A. & Braff, D.L. Startle habituation and sensorimotor gating in schizophrenia and related animal models. *Schizophr. Bull.* **13**, 643–668 (1987).
10. Thornton, C.E., Brown, S.D.M. & Glenister, P.H. Large numbers of mice established by in vitro fertilisation with cryopreserved spermatazoa: implications and applications for genetic resource banks, mutagenesis screens and mouse backcrosses. *Mamm. Genome* **10**, 987–992 (1999).
11. Nolan, P.M. *et al.* Heterozygosity mapping of partially congenic lines: mapping of a semi-dominant neurological mutation, wheels (*Whl*), on mouse chromosome 4. *Genetics* **140**, 245–254 (1995).
12. *Medical Research Council Responsibility In the Use of Animals for Medical Research* (Medical Research Council, London, 1993).
13. Nolan, P.M., Kapfhamer, D. & Bucan, M. Random mutagenesis screen for dominant behavioural mutations in mice. *Methods* **13**, 379–395 (1997).
14. Irwin, S. Comprehensive observational assessment: 1a. A systematic, quantitative procedure for assessing the behavioural and physiologic state of the mouse. *Psychopharmacalogica* **13**, 222–257 (1968).
15. Candy, C.J., Wood, M.J. & Whittingham, D.G. Effect of cryoprotectants on the survival of follicles in frozen mouse ovaries. *J. Reprod. Fert.* **110**, 1–19 (1997).