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Fierce: a new mouse deletion of *Nr2e1*; violent behaviour and ocular abnormalities are background-dependent

Kelly A. Young[a], **Melissa L. Berry**[b], **Connie L. Mahaffey**[b], **Jennifer R. Saionz**[b], **Norman L. Hawes**[b], **Bo Chang**[b], **Qing Yin Zheng**[b], **Richard S. Smith**[b], **Roderick T. Bronson**[c], **Randy J. Nelson**[d,1], and **Elizabeth M. Simpson**[e,*]

[a]Department of Biochemistry and Molecular Biology, Johns Hopkins School of Public Health, Baltimore, MD 21205, USA

[b]The Jackson Laboratory, Bar Harbor, ME 04609, USA

[c]USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA 02111, USA

[d]Departments of Psychology and Neuroscience and Biochemistry and Molecular Biology, Johns Hopkins University, Baltimore, MD 21218, USA

[e]Centre for Molecular Medicine and Therapeutics, British Columbia Research Institute for Children's and Women's Health, Department of Medical Genetics, University of British Columbia, 950 West 28th Avenue, Vancouver, BC, Canada V5Z 4H4

Abstract

A new spontaneous mouse mutation named fierce (*frc*) is deleted for the nuclear receptor *Nr2e1* gene (also known as *Tlx*, mouse homolog of *Drosophila tailless*). The fierce mutation is genetically and phenotypically similar to *Nr2e1* targeted mutations previously studied on segregating genetic backgrounds. However, we have characterized the fierce brain, eye, and behavioural phenotypes on three defined genetic backgrounds (C57BL/6J, 129P3/JEms, and B6129F1). The data revealed many novel and background-dependent phenotypic characteristics. Whereas abnormalities in brain development, hypoplasia of cerebrum and olfactory lobes, were consistent on all three backgrounds, our novel finding of enlarged ventricles in 100% and overt hydrocephalus in up to 30% of fierce mice were unique to the C57BL/6J background. Developmental eye abnormalities were also background-dependent with B6129F1-*frc* mice having less severe thinning of optic layers and less affected electroretinogram responses. Impaired regression of hyaloid vessels was observed in all backgrounds. Furthermore, retinal vessels were deficient in size and number in 129P3/JEms-*frc* and B6129F1-*frc* mice but almost entirely absent in C57BL/6J-*frc* mice. We present the first standardized behavioural tests conducted on *Nr2e1* mutant mice and show that C57BL/6J-*frc* and B6129F1-*frc* mice have deficits in sensorimotor assays and are hyperaggressive in both sexes and backgrounds. However, C57BL/6J-*frc* mice were significantly more aggressive than B6129F1-*frc* mice. Overall, this extensive characterization of the fierce mutation is essential to its application for the study of behavioural, and brain and eye developmental disorders. In addition, the background-dependent differences revealed will enable the identification of important genetic modifiers.

Keywords

Aggressive behaviour; Sensorimotor; Eye; Brain

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* Corresponding author. Tel.: +1-604-875-3830; fax: +1-604-875-3819. simpson@cmmt.ubc.ca (E.M. Simpson)..
[1]Present address: Departments of Psychology and Neuroscience, 9 Townsend Hall, Ohio State University, Columbus, OH 43210, USA.

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1. Introduction

The study of heritable behaviour has been impeded by an evolving code of human ethics and a relative lack of animal models for behaviour [3]. Recently, behavioural studies using mouse model systems have emerged based on the powerful mouse genetics of single gene mutations. Using mouse models, numerous genes have been reported to contribute to reproductive, maternal, ingestive, and social behaviours [2,29,31]. In addition, genes involved in serotonin metabolism [6,25], adrenergic alpha2C receptor, neuronal nitric oxide synthase [30], and enkephalins [23] have been implicated in aggressive behaviour in mice. These models have provided interesting insights into portions of behaviour that are influenced genetically.

To interpret the effects of a genetic lesion accurately, genetic background must be considered [6,13,33]. This may be particularly relevant for variable phenotypes such as behaviour studied in highly inbred strains recognized as having identifiable characteristics [6,24]. Firstly, studying behavioural mutants on controlled backgrounds using inbred or F1 animals allows mutants to be compared with otherwise genetically identical littermates. This strategy also allows for reproducibility in time and place. Secondly, the use of multiple backgrounds highlights genetic variability and more closely parallels the differences between human individuals. This is especially true of F1 hybrid animals that provide the genetic variability of a heterozygous state at all loci on a controlled, reproducible, and non-segregating background. In addition, phenotypic differences between strains can lead to the identification of genetic modifiers in mice, and ultimately, in humans. Thus, the applicability of mouse genetics is enhanced by phenotypic assessment on multiple, defined backgrounds.

Two targeted disruption mutations in mice have been described for the gene *Nr2e1* (nuclear receptor subfamily 2, group E, member 1), the mouse homologue of the *Drosophila* nuclear receptor *tailless* (also known as *Tlx*). These mutant mice have an extensive developmental defect of the anterior brain, aggressive behaviour [27], and severe impairment of retinal and optic nerve development [40]. However, a detailed characterization of the extent of aggressive behaviours has not been performed and this, along with brain and eye phenotypes, have only been described on mixed and segregating 129 and C57BL/6 genetic backgrounds. Furthermore, sensorimotor evaluation has not been performed on these mice—an evaluation of which is essential to the interpretation of other behaviours [5,29].

Here we report on a new mouse mutation named ‘fierce’ (*frc*) that includes a spontaneous deletion of *Nr2e1*. The *frc* mutation has been studied on three commonly used genetic backgrounds, C57BL/6J, 129P3/JEms, and B6129F1, to comprehensively examine behaviour, brain, and eye phenotypes [9,34]. Whereas many of the elements of the fierce phenotype are consistent on all three genetic backgrounds indicating their fundamental importance, we have identified some elements that are background-dependent, thus highlighting the genetic heterogeneity and potential for the identification of modifier genes in aggressive behaviour and brain development. In addition, we conducted a panel of sensorimotor, aggression, and mating tests on C57BL/6J and the hybrid B6129F1 mouse backgrounds. Such detailed behavioural characterization increases the value of this important mouse model permitting further inquiry not only into the consistent phenotypes of brain development and aggression but also into traits exposed on genetically distinct mouse backgrounds.

2. Methods

2.1. Mouse backgrounds

The *frc* mutation was not deliberately induced and thus, is categorized as a spontaneous mutation. It was recovered during a targeted mutagenesis experiment in which a null allele of

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the zinc finger protein, autosomal (*Zfa*) was derived using 129E14TG2a embryonic stem cells (from 129P2/OlaHsd mice [18]). Chimeras made by injecting the ES cells into C57BL/6J (JAX®, 000664) embryos were crossed to C57BL/6J mice. A single germline hybrid animal was used to initiate the backcrossing to generate C57BL/6J and 129P3/JEms (JAX®, 002357) congenic strains. Note: 129P3/JEms is the closest available relative to 129P2/OlaHsd [34]. Segregation of the *Zfa* and *frc* mutations occurred independently during the backcrossing to both C57BL/6J and 129P3/JEms resulting in the generation of four congenic strains; C57BL/6J-*frc*, C57BL/6J-Zfa^{tmlEms}, 129P3/JEms-*frc*, 129P3/JEms-Zfa^{tmlEms}. Here we report only on the characterization of the fierce mutants.

Animals for experimental analysis were generated by crossing heterozygotes such that controls and mutants are littermates. In accordance with Mendelian inheritance, 25% of the offspring are homozygous mutants. For the C57BL/6J background, animals studied were incipient congenics ranging from generation N6 to N7 (98.4–99.2% C57BL/6J), for 129P3/JEms they were N6 (98.4% 129P3/JEms), and for B6129F1 they were the offspring of C57BL/6J females (N6–N11, 98.4–99.9% C57BL/6J) mated to 129P3/JEms males (N5–N8, 96.9–99.6% 129P3/JEms). Some early experiments were conducted on C57BL/6J animals at N3 (87.5% C57BL/6J: body weights, fat pads, testosterone, and brain morphology) and on 129P3/JEms animals at N4 (93.8% 129P3/JEMS: brain morphology). We observe no changes in the reported phenotypes in these animals, now congenic at >N10 (>99.9% pure). To date we have not identified a heterozygous phenotype in any of the backgrounds studied, indicating that the *frc* mutation is recessive. Thus, for this study, controls were defined as wild-type or heterozygous mice.

2.2. Southern and northern blots

A Southern blot of *Bgl*II-digested mouse DNA (from B6129F1 of N6 parents) was hybridized as previously described [35] with a probe for zinc finger autosomal (*Zfa*) (1.4 kb *Ssp*I genomic fragment from pEMS229), and then *Nr2e1* (1078 bp reverse transcription-polymerase chain reaction (RT-PCR) product from embryonic brain including almost all the coding region using oEMS294 TTGCAAAGTGTGTGGTGACC and oEMS295 TCTTGTAATCGGCACATTGC, subsequently cloned as pEMS741). A northern blot of total RNA from adult liver, brain, and testis (from B6129F1 at N6) was hybridized with the same *Nr2e1* probe and sequentially with a human *GAPDH* probe (pHcGAP 1.25 kb *Pst*I cDNA fragment [38]).

2.3. Growth experiments

Body weight of C57BL/6J mice (N3) was recorded from embryos at e12.5, newborns, and then weekly from 1 to 10 weeks of age from females (ten *frc*, 27 controls), and males (nine *frc*, 24 controls). Body weight was also recorded from *frc* and control 3-week-old mice from all three backgrounds at N6 (C57BL/6J n = 86, 129P3/JEms n = 88, B6129F1 n = 143). Total fat was analyzed from 3-month-old C57BL/6J mice at N3 (four *frc*, eight controls). Fat pads were removed and weighed from gonadal, inguinal, mesenteric, and peritoneal regions [37]. Total fat was the sum of all fat pads from each animal.

2.4. Brain morphology

Mice were injected with a lethal dose of tribromoethanol (Avertin), flushed with saline, and perfused with Bouin's fixative. Brains of 24 C57BL/6J at N3 (two *frc* and four controls from each of four time points: 1 week, 1, 3, and 6 months), ten adult 129P3/JEms at N4 and 28 B6129F1 at N6 (seven adults, ten 1-week old, 11 newborns) mice were processed for serial sectioning and stained with hematoxylin and eosin (H and E), or Luxol fast blue/Cresyl violet (LFB-CV). Sections from the 3-month-old C57BL/6J mice were also stained for glial fibrillary acidic protein (GFAP) using an avidin–biotin-labeled immunoperoxidase and a GFAP

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antibody (Sigma, St. Louis, MO). Brains of B6129F1 embryos at N6–N10 (13 at e16.5, nine at e14.5, and 16 at e12.5) were harvested immediately from CO_2-euthanized mothers and stained with H and E.

2.5. Retinal histology

Eyes were removed immediately from CO_2-euthanized mice, placed in Telly's fixative, and processed routinely for H and E sections from 18 mice, 11–12 weeks old (three *frc* and three littermate controls from all three backgrounds at N6–N7). In addition, H and E sections were examined from three *frc* and three littermate controls at e16.5 from B6129F1 (N10 and N8).

2.6. Retinal morphometry

The thickness of the retinal layers was measured in one eye from 22 mice 11–12 weeks old at N6–N7 (12 controls: C57BL/6J, two males, two females; 129P3/JEms, two females; B6129F1, four females, two males. Ten fierce: C57BL/6J, one male, one female; 129P3/JEms, two females; B6129F1, two females, four males). The plane of section studied included the optic nerve and six measurements were made from a standardized location in each eye; three temporal and three nasal of the nerve. (1) The full thickness of the retina from internal limiting membrane to apex of retinal pigment epithelium. (2) The inner nuclear layer from innermost to outermost nucleus. (3) The photoreceptor nuclear layer from innermost nucleus to external limiting membrane.

2.7. Electroretinograms

Electroretinograms (ERGs) were performed on 7–12 week old control and *frc* mice from all three backgrounds (C57BL/6J and 129P3/JEms at N6, B6129F1 at N7–N10) as previously described [15]. Briefly, prior to dark adaptation of 60 min, the eyes were dilated with 1% topical atropine. Once dark adapted, mice were anesthetized typically with a mixture of 0.46 mg xylazine and 0.72 mg ketamine injected subdermally. Reference and ground were standard EEG needle electrodes placed subdermally over the mandibles of the anesthetized mouse, while the active electrode was a platinum wire (Grass E2, Quincy, MA) placed so it was gently touching the superior cornea. Several intensities were used to stimulate the evoked response using the Grass photostimulator and standard neutral density filters.

2.8. Behavioural testing

2.8.1. Behaviour tests—Twenty-four control and three fierce males, and 20 control and five fierce females of the C57BL/6J background (N6) and 11 control and 11 fierce males, and six control and six fierce females of the B6129F1 background (from N6–N10 mothers and N6–N8 fathers) were evaluated for behaviour. Within each background, mice were either littermates or close relatives. All animals were coded with randomized experimental numbers although the ‘hard to handle’ phenotype made it difficult to conduct a completely blind study. Mice were 80±16 days of age at the onset of testing, and were housed individually in polycarbonate cages (28×17×12 cm) with ad libitum access to both tap water and food, except where noted (Agway Prolab 2000, Syracuse, NY). A 16:8 h light:dark cycle was maintained for the duration of the study (lights on at 07:00 h Eastern Standard Time), with ambient temperature kept at 21±2 °C and relative humidity 50±5% throughout the behavioural study. All animals completed the full panel of tests.

2.8.2. Sensorimotor tests—The following standardized sensorimotor tests were performed on three separate trials as previously described [22] and were completed prior to the onset of aggression and mating tests. Olfactory ability was assessed with the Find the Hidden Cookie test conducted immediately after a 12 h (C57BL/6J) or 6 h (B6129F1) period of food deprivation. A chocolate chip cookie was hidden beneath 3–6 cm of wood shavings in an

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aquarium. Each mouse was released into the aquarium and the time required to locate the cookie was recorded for a maximum of 10 min. Vision and depth perception were assessed with the Visual Placing test: mice lowered towards the edge of a table by their tail were assigned a positive score if they extend their forepaws prior to touching the table on >2 of three trials. Gait, locomotion, agility, and motor coordination were assessed with the combination of tests: Turning in a Blind Alley, and Turning on an Inclined Screen. In the former, the mouse was placed facing the back wall of an alley (3-cm wide, 35-cm long, with walls 15-cm high). The amount of time for the mouse to turn around and face the open end of the alley was recorded. In the latter, the mouse was placed at the center of a wire mesh screen (35 cm^2) that was tilted at a 45° angle approximately 75 cm above a large pillow. The mouse was placed on the screen facing downward, and the time taken by the mouse to turn to face upward was recorded. In both tests, the time to turn was recorded in a 2-min period. Exploratory behaviour was assessed with the Elevated Plus Maze test: mice were placed in the center of an elevated plus maze with two open arms (67×5.5 cm) and two closed arms (67×5.5 cm with 15-cm high black-tinted plexiglass walls and a 65-cm removable roof) placed 75 cm above the floor on a tripod. The number of visits to each arm and the time spent in each arm, as well as in the central area were recorded for 5 min [5]. Hearing/auditory function was assessed by the Click and Tone-Pip Stimuli test [19].

2.8.3. Aggression tests—Aggression and mating tests were performed as previously described [22]. Male and female experimental mice in their home cages were presented with a control same-sex adult mouse in the Aggression Against Intruder in Home Cage test. Male experimental mice were presented with a control adult male in a neutral cage in the Aggression in a Neutral Arena test. In both tests, the duration of each hostile encounter, and the number of aggressive encounters during a 15-min test was recorded. The test was immediately aborted if a mouse became injured. If injured, mice were removed and had their wounds treated and dressed daily until healed. A unique pairing was made for each test.

2.8.4. Mating tests—Males of the C57BL/6J background were allowed to acclimate to a home cage for 5 days prior to testing. Control females were primed with 20 μg of 17-β-estradiol 48 h prior to testing, and 500-μg progesterone 5 h prior to testing. Females that displayed lordosis were selected using stud males, and then individually introduced to the mating arena with the experimental male. Latency to begin mounting behaviour, the number of mounts, and the number of aggressive encounters towards females were recorded as previously described [30].

2.9. Testosterone and corticosterone RIA

Coritcosterone levels were measured on all mice used for behaviour testing. Blood samples were collected via retro-orbital bleeding into iced heparinized tubes from lightly anesthetized mice 2 days before and 2 days after all behavioural testing. Plasma was stored at −80 °C so that all samples could be processed in a single batch. Corticosterone concentrations were assayed in duplicate by RIA ^{125}I kits (ICN Biomedicals, Costa Mesa, CA). Testosterone levels were measured on C57BL/6J mice at N3 (males: eight *frc* and 16 controls; females: five *frc* and ten controls). Blood collection and serum assay were performed as previously described [1].

2.10. Data analysis

Statistical evaluation of mean differences between fierce and control mice were performed by *t*-tests, or, for measurements lacking equal variance, by a Mann–Whitney Rank Sum Test on Ranks using the Sigma Stat software package (Jandel Scientific, San Rafael, CA) or the JMP statistical software (SAS, Cary, NC). Males and females were analyzed separately. Mean differences were considered statistically significant when $P<0.05$. Differences not found to be

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significant were not interpreted if the tests had less than 80% power. Analyses of retinal thickness between all three backgrounds were done with an ANOVA or, for measurements lacking equal variance, with Kruskal–Wallis ANOVA using the JMP. Data are reported as mean values±1 S.E. of the mean.

3. Results

3.1. The fierce mutation includes a deletion of the nuclear receptor Nr2e1

We have developed a mouse mutation named fierce (*frc*) due to its dramatically violent behaviour. Preliminary characterization also revealed that *frc* mice had stunted growth, hypoplasia of the forebrain regions, and lack of maternal instinct. The *frc* mutation is a spontaneous mutation recovered during a targeted mutagenesis experiment of the zinc finger protein, autosomal (*Zfa*). Although the phenotypic characteristics were originally attributed to *Zfa*, analyses of subsequent generations showed segregation of the *Zfa* targeted allele and the *frc* mutation as defined by the 'hard to handle' behaviour. The independence of the fierce phenotype from the *Zfa* null allele is demonstrated in Fig. 1A where mice unaffected for handling behaviour are homozygous mutant for *Zfa* and a mouse affected for handling behaviour is wild type at the *Zfa* locus. The *frc* and *Zfa* mutations have been bred apart and are now maintained in separate mouse colonies. Subsequent genetic analyses have confirmed consistently the association between the *frc* mutation and the handling behaviour. Because the fierce mutation was an unknown spontaneous mutation, we initiated a candidate gene search for the responsible locus. Included in this search was the *Nr2e1* gene, since mice targeted for this locus show a reduced body weight, cerebral and olfactory hypoplasia, lack of maternal behaviour, and aggression [27]. As expected, the phenotypic similarities turned out to be more than coincidental; fierce includes a deletion of the coding region of *Nr2e1*. The Southern blot presented in Fig. 1B shows that mice affected with abnormal handling behaviour are homozygous deleted for *Nr2e1*, but unaffected mice maintain the *Nr2e1* gene. Northern blots confirmed the lack of an *Nr2e1* transcript in the brains of *frc* mice, but strong expression in the brains of control littermates (Fig. 1C and D).

3.2. Two congenics permitted comparison of three genetic backgrounds

Each of the inbred strains used (C57BL/6J or 129P3/JEms) had advantages and disadvantages for the study of this mutation. C57BL/6J-*frc* mice were highly aggressive but developed hydrocephalus with a frequency that increased with increasing backcross generation, eventually leading to 30% mutant animals with overt hydrocephalus. Such mice do not make an ideal model for the study of other brain abnormalities. In contrast, the 129P3/JEms-*frc* mice showed little or no hydrocephalus but had such small litters that it was difficult to produce sufficient animals for behavioural studies. We, therefore, developed B6129F1-*frc* hybrids by breeding C57BL/6J-*frc* heterozygous females to 129P3/JEms-*frc* heterozygous males. This breeding scheme produces C57BL/6J-like sized litters, and the F1 hybrid mutant animals showed little or no hydrocephalus. F2 animals, produced from F1 × F1 crosses, have mixed, segregating backgrounds, and thus, were not studied. Hence, we generated three genetic backgrounds for study from two congenic strains [33].

3.3. Fierce mice have stunted growth parameters

Fierce mice are, on average, smaller than their control littermates just as described for *Nr2e1* targeted mutant mice [27]. This observation was true for both male and female fierce mice (Fig. 2A and B). The growth differential is not present in embryos or in newborn pups but develops between postnatal weeks 2 and 3. Having either not gained or, in some cases, lost weight during this period, on average, the mutant animals remain smaller than littermates as adults. In addition, as a percentage of total body weight, the mean total fat pads from *frc* mice was significantly less than those from controls (for C57BL/6J: *frc* 3.1±1.0% vs. control 5.7

±2.0%). Although some of the difference in weight is attributable to the fact that the *frc* mutants are generally leaner than their littermates, the mutants also show a trend to be smaller on average from nose to rump. These observations were true for mice from all three backgrounds although the C57BL/6J-*frc* mice were significantly more affected than the B6129F1-*frc* mice; mean weight at 3 weeks was 6.0±0.3 g for B6129F1, and 8.2±0.3 g for B6129 (P<0.001, Fig. 2C).

3.4. Fierce mice have abnormal brain development

Histopathological examination of the brains from *frc* mice identified hypoplasia and distortion of the anterior aspects of the brain in mice from all three backgrounds. In a mouse with a mild reduction in size of the whole animal, it is not normal to see reduction in brain size [32,36]. In fact, whereas the cerebellar regions in *frc* mice were not different from controls, the cerebrum and olfactory regions of the fierce brain were disproportionately reduced (Fig. 3A). The fierce cerebrum appeared flattened and did not overhang the rostral colliculi. Despite gross hypoplasia, no nuclei or tract was completely absent in homozygous fierce brains. The rostral commissure was almost always thinner in *frc* mice. In the severe example shown, this commissure was small throughout its course and was particularly thin and wispy as it crossed the midline (Fig. 3B and D). Overall, the developmental brain abnormalities in adult mice homozygous for *frc* were similar to those described for mice homozygous for the targeted null allele of *Nr2e1* [27].

We extended our studies to determine whether the brain phenotype was due to degenerative or developmental abnormalities. As an assessment of neural degeneration, immunohistochemical staining for GFAP was performed on sections from 3-month-old mice. The brains of all mice stained with no evidence of reactive gliosis. A histological comparison of LFB-CV sections from *frc* brains at different time points revealed no obvious difference between 3 and 6 months of age. The distinctive morphology evident in adult *frc* mice was also present in *frc* brains at 1 month and 1 week of age, although less prominent in the lesser developed brain. Examination of newborn and embryonic B6129F1-*frc* brains revealed that the pathological changes were observed consistently in newborns (Fig. 3C, D and E), and hypoplasia of the anterior brain was evident even in embryos at e16.5 (Fig. 3F) but not at e14.5 or e12.5. This observation is consistent with the expression of *Nr2e1* in the developing brain, which peaks at e13 [28]. Taken together, the lack of GFAP staining and the patterning of pathological changes that parallels development suggest that *frc* causes a developmental abnormality and not degeneration of a normally developed brain.

A novel observation in the C57BL/6J-*frc* mice was an increased risk of hydrocephalus with as many as 30% of *frc* mice displaying overt hydrocephalus and all *frc* mice showing enlarged ventricles. In contrast, few, if any, of the *frc* mice had enlarged ventricles on the 129P3/JEms or B6129F1 backgrounds.

3.5. Fierce mice have impaired ocular development

The retinas of all *frc* mice studied showed abnormalities compared with littermate controls. The *frc* retinas showed either a poorly defined or absent nerve fiber layer and thinner inner nuclear and photoreceptor layers than the controls (three cells thick vs. eight to ten cells). In addition, the ganglion cell layer showed incomplete differentiation, with cell nuclei remaining in the inner plexiform layer resembling the developing retina characteristic of a day 14–15 embryo (Fig. 4A). Furthermore, the optic nerves were smaller in diameter and the architecture was disrupted with collapse of the pial septa due to the absence of optic nerve axons. Since the nerve fibres of the optic nerve arise from the retinal ganglion cells, the two observations are likely to be related [16]. These ophthalmic changes are consistent with those described for an *Nr2e1* targeted mutant mouse [40]. However, a novel observation from the studies of retinas from B6129F1-*frc* day 16.5 embryos showed that, although the retinal thickness looked normal,

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the inner retinal layer showed retarded separation into bipolar and ganglion cell layers. This observation likely explains the misplaced cells in the inner plexiform layer of adult *frc* mice and suggests that the eye phenotype is, at least partly, developmental.

To determine the effects of background on this phenotype, we measured the thickness of retinal layers and optic nerve in male and female *frc* and control mice from all three backgrounds. In all three backgrounds, *frc* mice showed thinner layers than control mice. This was significant for all six layers in C57BL/6J mice (P<0.003) but in only two layers for the 129P3/JEms mice (photoreceptor temporal P<0.04, full nasal P<0.02) and four layers for B6129F1 mice (bipolar temporal P<0.02, photoreceptor temporal P<0.002, bipolar nasal P = 0.05, and photoreceptor nasal P<0.006). The C57BL/6J mice had a trend for all retinal layers to be thicker than in the other two backgrounds. This reached statistical significance for the bipolar nasal layer in fierce mice (P<0.03) and for four of the layers in control mice (full temporal P<0.007, photoreceptor temporal P<0.03, full nasal P<0.008, bipolar nasal P<0.03).

Impaired regression of the hyaloid vascular system of the eye was a novel observation in *frc* mice on all three backgrounds. These blood vessels, present in the developing mouse, normally start to involute between post-natal day 5–10 and disappear completely by 1 month of age [20]. However, in adult *frc* mice on all three backgrounds, large and small vascular channels were present in the vitreous, close to the optic nerve (Fig. 4A) and the peripheral retina. It appeared that some vessels were coming directly out of the retina into the vitreous rather than via central vessels. Since the embryonic hyaloid vascular system never develops vessels of this diameter, it seems likely that these are hyaloid vessels that have failed to undergo apoptosis and have, perhaps, enlarged. There was no evidence for a retinal origin of these vessels. In contrast to the extraneous vessels in the vitreous, *frc* mice lacked a normal pattern of retinal vessels. Examination of the fundus of the eyes revealed that retinal vessels were small in size and few in number in all 129P3/JEms-*frc* and B6129F1-*frc* mice studied but were mostly absent in C57BL/6J-*frc* mice (Fig. 4B).

The electroretinograms (ERGs) of adult *frc* mice were consistently abnormal on all three backgrounds (Fig. 4C). The ERG signal was non-detectable with single flash methods in C57BL/6J-*frc* mice, supporting the phenotype of the *Nr2e1* targeted mutant [40]. An identical profile was found with *frc* mice from the 129P3/JEms background. However, background-dependent variation was apparent for B6129F1-*frc* mice whose ERGs demonstrated a b-wave amplitude of 207 V (~25% of normal) in all six animals studied.

3.6. Auditory function

No deficiencies in auditory-evoked brainstem response were detected in 4-month-old C57BL/6J-*frc* male mice using click and tone-pip stimuli [21]. Both heterozygotes and homozygotes were compared with standard hearing for C57BL/6J [41] and no differences were detected (data not shown).

3.7. Fierce mice lack maternal behaviour, are ‘hard to handle’ and violently aggressive

Female *frc* mice lack maternal behaviour, often choosing to abandon their nest rather than nurse their pups. Furthermore, both male and female *frc* mice display a ‘hard to handle’ phenotype, responding to being held by the tail by vocalizing, struggling, jumping and biting. These behaviours are identical to those described for the targeted mutant mouse [27]. In addition, these behaviours are consistently expressed in mice from all three backgrounds; the lack of maternal behaviour is less evident on the 129P3/JEms and B6129F1 backgrounds. We also observed that the B6129F1-*frc* mothers gave birth to the same number of pups as controls but that the lack of maternal behaviour caused a reduction in the number of pups surviving to wean (average # pups weaned: 9.2±1.5 vs. 5.8±0.7, P<0.009 for control vs. *frc*, respectively). In

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general, C57BL/6J-*frc* female mice had decreased fertility, presumably due to the presence of hydrocephaly. However, among successful matings, female *frc* mice had the same number of pups as controls, none of which survived to wean (average # pups weaned: 5.8±1.3 vs. 0, $P<0.02$ for control vs. *frc*, respectively).

More extraordinary than the 'hard to handle' phenotype, was the violent (i.e. drawing blood) aggressive behaviour displayed by *frc* mice starting at about 5 weeks of age. Despite their smaller stature, *frc* males routinely and repeatedly attacked their siblings, occasionally leading to death if the *frc* males were not separated. Male violence was also directed toward mating partners. In a retrospective analysis of initial breeding experiments designed to test fertility in *Zfa* C57BL/6J mutants, females in every mating were either wounded or killed. These devastating results have precluded the use of *frc* males in any subsequent mating experiments. Although aggression to this degree is occasionally seen in certain rare inbred strains, we have never seen it in C57BL/6J, 129P3/JEms, or B6129F1 backgrounds or in control littermates from these *frc* colonies. We, therefore, conducted standardized testing of sensorimotor skills and aggressive behaviour.

3.8. Fierce mice show deficits in standardized sensorimotor tests

The lower productivity of the 129P3/JEms strain rendered it impractical to obtain quantities of age-matched *frc* mice and so sensorimotor and behaviour tests were only conducted on the C57BL/6J and B6129F1 backgrounds (Table 1). Female *frc* mice from both backgrounds showed diminished but functional ability to find the hidden cookie but took longer than controls to sniff and nibble it. Likewise, male C57BL/6J-*frc* mice took longer than controls to sniff the cookie (Fig. 5A). Male C57BL/6J-*frc* mice also showed deficits in visual placing compared with controls. When lowered to the edge of a table, *frc* mice extended their paws less often, scoring an average of 0.22±0.2 compared with 0.72±0.1 for controls ($P = 0.04$).

All four groups of *frc* mice showed some degree of deficiency in motor tests (Table 1). Male *frc* mice on both backgrounds took three times longer than controls to turn on an inclined screen, as did female B6129F1-*frc* mice. Female C57BL/6J-*frc* mice took longer than controls to turn in an alley.

Differences in exploratory behaviour in *frc* mice were noted in the elevated plus maze test (Table 1; Fig. 5B). Male C57BL/6J-*frc* mice showed a preference for the unprotected open arms spending more time and more visits than controls. Moreover, male *frc* mice on both backgrounds avoided the closed arms compared with controls. B6129F1-*frc* males made fewer visits to closed arms than controls while C57BL/6J-*frc* males spent less time in closed arms than controls (Fig. 5B). This observation was also true for female C57BL/6J-*frc* mice. In inter-background comparisons, the time spent in closed arms by male *frc* mice was less in C57BL/6J mice than in B6129F1.

3.9. Aggressive behaviour is background-dependent in standardized tests

Male C57BL/6J-*frc* and B6129F1-*frc* mice were more aggressive than controls in home cage tests (C57BL/6J $P<0.02$, B6129F1 $P<0.001$, Fig. 6A). Male C57BL/6J-*frc* mice were also more aggressive than controls in neutral arena tests ($P<0.01$, Fig. 6B). In inter-background comparisons, *frc* males from the C57BL/6J background were more aggressive than those from B6129F1, attacking their intruders more often in both the home cage ($P<0.002$) and neutral arena ($P<0.03$) encounters. Importantly, this inter-background difference was not seen in control mice.

Standard tests for female aggression are based on nest defense [12]. However, for these mutants, where a lack of maternal care is observed, the assessment of aggression during nest

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defense seemed inappropriate. Instead, an intruder paradigm equivalent to that used for male mice was employed with female intruders and residents. Aggressive behaviour was prominent in the female C57BL/6J-*frc* mice who engaged in 96% more aggressive attacks than controls in their home cage test ($P<0.001$, Fig. 6A). These females initiated all attacks, and persisted despite obvious displays of submission observed in the control mice. And, as observed for the males, inter-background differences were significant as female C57BL/6J-*frc* mice attacked intruders more often than did B6129F1-*frc* mice ($P<0.001$). This inter-background difference was not seen in the control mice.

3.10. Fierce mice show abnormal behaviour in mating tests

Latency to initiate mounting behaviour towards hormonally primed females was significantly longer in *frc* mice than in controls (1800±0.0 vs. 473±21.5 s, $P<0.05$). The total number of mounts was significantly less in the *frc* males than in the controls (0 vs. 21.5±4.1, $P<0.05$). During mating tests, *frc* mice engaged in ten-fold more aggressive encounters towards females than controls (10.7±8.3 vs. 1.1±0.6).

3.11. Corticosterone and testosterone concentrations are not abnormal in fierce mice

Concentrations of corticosterone from C57BL/6J and B6129F1 mice did not differ between backgrounds or between *frc* mice and sex-matched littermate controls. Testosterone concentrations from C57BL/6J mice did not differ between male *frc* and control mice (>80% power to detect a difference of 2.5 ng/ml at $P = 0.05$). No testosterone was detected above 0.1 ng/ml for female mice. All hormone concentrations fell within the standard range (Table 2).

4. Discussion

We have identified a novel spontaneous mouse mutation named ‘fierce’ that includes a deletion of *Nr2e1*. From phenotypic comparisons and molecular analyses, we conclude that the *frc* mutation is very similar to that introduced in *Nr2e1* targeted mutant mice [27,40]. Further characterization of *frc* mice has identified novel phenotypes for this lesion that have not been reported for *Nr2e1* targeted mutants. These include decreased body fat, increased incidence of hydrocephaly on the C57BL/6J background, failed regression of the hyaloid vasculature, absence of normal patterns of retinal vessels and retarded development in the embryonic retina. These distinct traits are most likely to be observed because of the extent of phenotypic characterization, the effects of genetic background, or both. Although we cannot exclude the possibility that the *frc* mutation includes genetic alterations beyond *Nr2e1*, preliminary data suggest the deletion is small (<50 kb) (unpublished data).

The importance of phenotyping genetic lesions on multiple backgrounds is three-fold. Firstly, the mere isolation of the *frc* mutation proffers the warning that genetic purification through backcrossing is imperative to associate genetic lesions with phenotypic outcome. Without the appropriate degree of backcrossing, the *frc* ‘hard to handle’ phenotype would have been inappropriately attributed to the targeted *Zfa* allele. Secondly, the identification of traits consistent on all backgrounds studied is suggestive of the fundamental functions of the gene that are likely to be consistent across species. Thirdly, through genetic purification, background-specific modulations of *frc* traits have been revealed. Such variable phenotypes in mice are important for the identification of potential genetic modifiers and as predictors of syndromes in humans.

A prominent background-dependent phenotype was overt hydrocephalus seen only in *frc* mice on the C57BL/6J background. In contrast, all other developmental brain abnormalities, as well as stunted growth, were consistent across all three backgrounds studied. These observations

support the known predisposition of C57BL/6J inbred mice to hydrocephalus of 1–3% [7,8] and the known expression of *Nr2e1* in brain development [28].

Background-dependent phenotypes were also observed among the ophthalmic abnormalities. On both inbred backgrounds studied, C57BL/6J and 129P3/JEms, the *frc* mutation resulted in more pronounced optic lesions than that found in the hybrid background, B6129F1. Furthermore, ERG testing showed the most diminished transmission in *frc* mice from the C57BL/6J and 129P3/JEms backgrounds. Visual placing tests of the C57BL/6J versus B6129F1 backgrounds paralleled these ERG results. Since the retina develops as an outgrowth of the anterior brain and *Nr2e1* is expressed in the anterior brain and optic cup during development, the developmental defects in *frc* retina, optic nerves, and optic vessels are not surprising [17,28,40]. The role of *Nr2e1* as a nuclear receptor important in eye development is well known [17]. However, the mechanism of action of *Nr2e1* is not understood and our novel observation of failed regression of the hyaloid vasculature and absence of normal pattern of retinal vessels in *frc* mice adds new perspective to the role of *Nr2e1* in eye development.

Background-dependence was not a prominent feature in sensorimotor tests. In both backgrounds, *frc* mice showed normal auditory function consistent with the fact that the ear develops from the hindbrain, a structure that normally lacks *Nr2e1* expression [28]. In both backgrounds, *frc* mice showed functional, albeit reduced, olfactory abilities although we cannot exclude the possibility that differences in motivation may con tribute to the mice's ability to find a hidden cookie. Mating and suckling are olfactory-dependent activities [26]; although the *frc* mice can do both, neither is entirely normal. Furthermore, olfactory abilities are critical for maternal location of pups [5]. Thus, the olfactory deficit may play a role, albeit minor, when compared to the central nervous system defects, in these behaviours. Although the *frc* mice are blind or have reduced vision, this impairment is not likely to contribute significantly to a lack of maternal behaviour as other blind mice are known to raise their young appropriately [11]. The avoidance of the protection of closed arms in the ‘elevated plus maze test’ by *frc* mice from both backgrounds could reflect either a reduced anxiety behaviour or simply, a lack of avoidance of the open arms due to vision defects. Behavioural abnormalities must also be considered when interpreting deficits in motor coordination, as the increased time taken by *frc* mice to turn on an inclined screen and in a blind alley could not be explained by any obvious abnormalities in gait or posture.

Standardized aggression tests revealed significant differences between backgrounds. Although *frc* mice from both backgrounds were more aggressive than controls in all comparisons (and reached significance in most), *frc* mice from the C57BL/6J background were always more aggressive than those from the B6129F1 background. The striking difference between these two backgrounds illustrates the critical importance of background on phenotype assessment and offers a means of identifying genetic modifiers of aggression. The aggression and differences in aggression between backgrounds are not the result of reduced or differing vision. Other strains of blind mice show that impaired vision and aggression are not linked [10]. However, the association between aggression and reduced olfaction is more complex, making a role for olfaction difficult to exclude completely. Aggression in female mice is rare. Although some targeted mutant strains have been characterized as more aggressive than their wild-type littermates, the increased aggression is often only observed in adults and only in the males [4,30]. Rarely is offensive aggressive behaviour observed outside of maternal nest defense in females from the same mutant strain [12]. Although it may appear that *frc* female behaviour has been masculinized, we detected no increase in testosterone in either sex and females become pregnant and have same number of pups as wildtype mice. Thus, the major cause for the aggression in *frc* mice is most likely the direct effect of the mutation on brain regions known to mediate aggression as demonstrated by septal region lesions in ‘rage’ mice and rats [14, 39].

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In conclusion, the *frc* mouse model provides a tool not only for studies in brain development, metabolism, violent aggression, and maternal behaviour, but also for identifying genes important in hydrocephaly, ocular development, and sex-limited modulation of aggression in females. This mutant will also serve as a tool to explore unusual events that may complicate targeting experiments. Finally, the detailed phenotypic characterization between backgrounds reveals similarities to elucidate fundamental roles for *Nr2e1* and differences that may reveal genetic modifiers of aggression, thus demonstrating a complex genetic syndrome.

Acknowledgments

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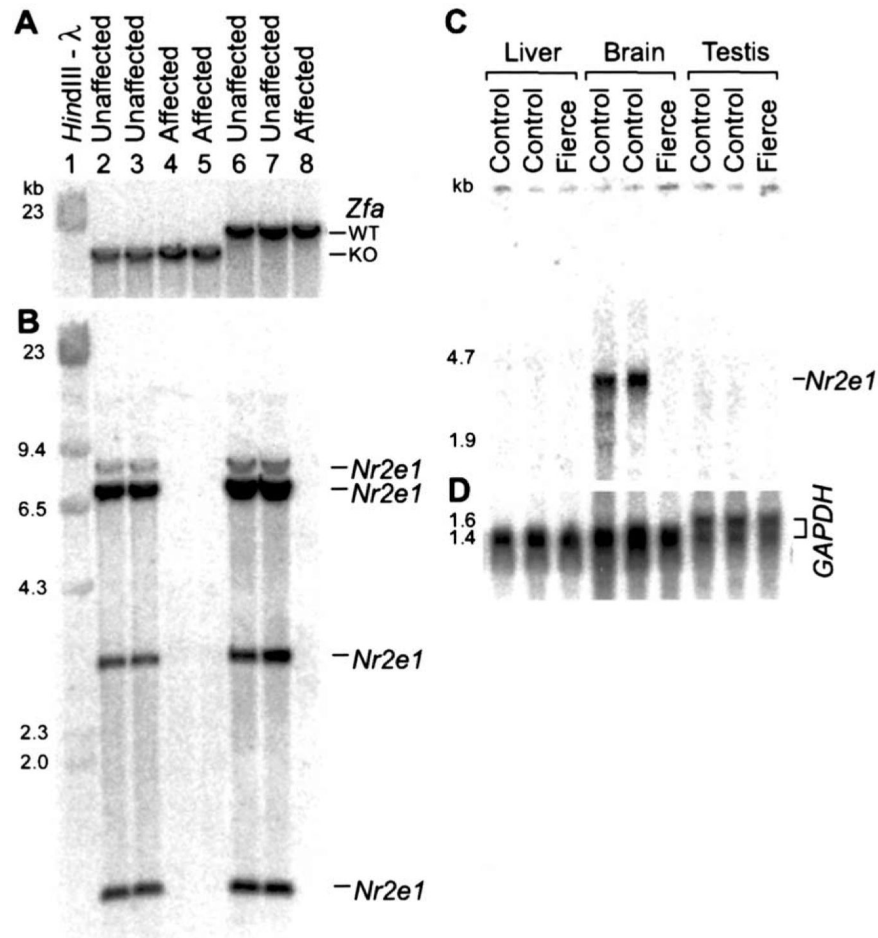

Fig. 1.

(A and B) Southern blot analyses of genomic DNA from mice show that the ‘hard to handle’ behaviour is associated with deletion of *Nr2e1*, not *Zfa*. (A) Hybridization with *Zfa* probe demonstrates unaffected *Zfa* mutant (KO) animals (lanes 2 and 3) and one affected wild-type (WT) (lane 8). (B) Same Southern hybridized with *Nr2e1* probe demonstrates that the gene is deleted in all affected mice and is present in all unaffected mice studied. (C) Northern analysis of adult total RNA shows that fierce brain lacks the *Nr2e1* transcript present in control littermates. The *Nr2e1* transcript is absent in two control tissues, liver, and testis. Size markers are 28S and 18S RNA. (D) Same northern hybridized with *GAPDH* as quantitative control.

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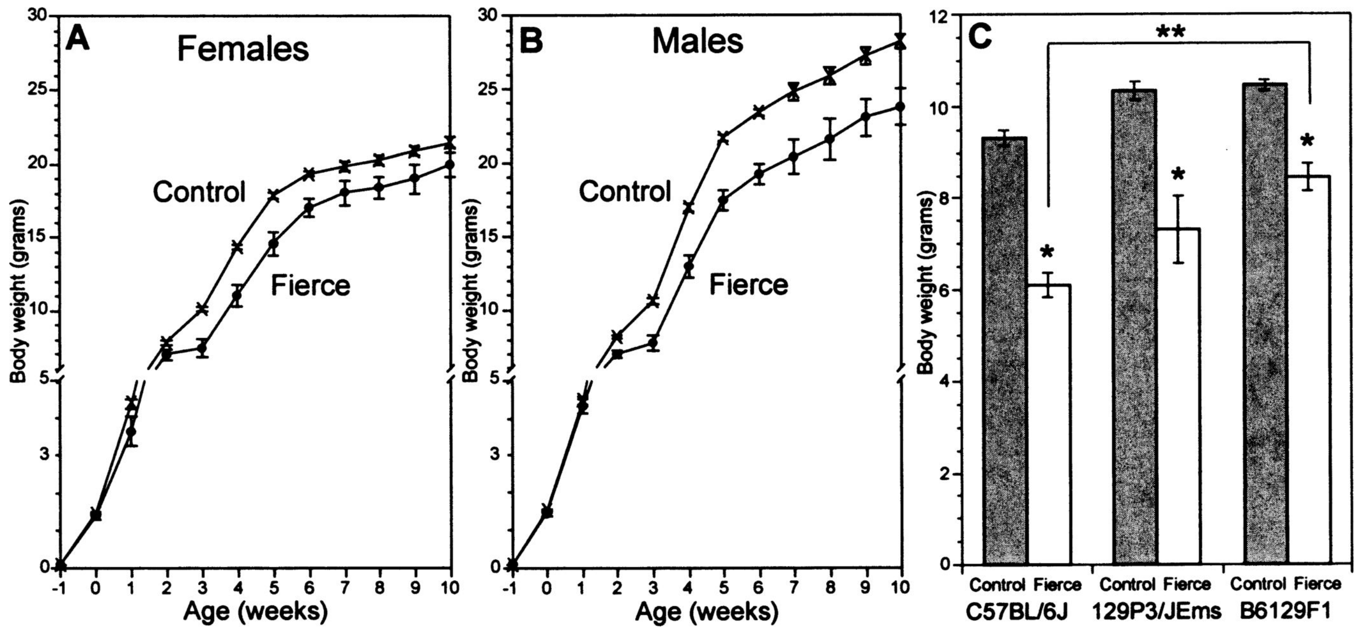

Fig. 2.

Fierce mice show postnatal growth failure. (A) Female and (B) male *frc* pups born of heterozygous C57BL/6J-*frc* parents fail to gain weight at the rate of their littermates, beginning at week 2. Age −1 is e12.5 and 0 is birth. (C) All three genetic backgrounds show smaller homozygous mutant mice at 3 weeks of age; data from both sexes are pooled. *, $P<0.05$. C57BL/6J: 25 *frc*, 61 controls; 129P3/JEms: 14 *frc*, 74 controls; B6129F1, 36 *frc*, 107 controls. **, $P<0.001$.

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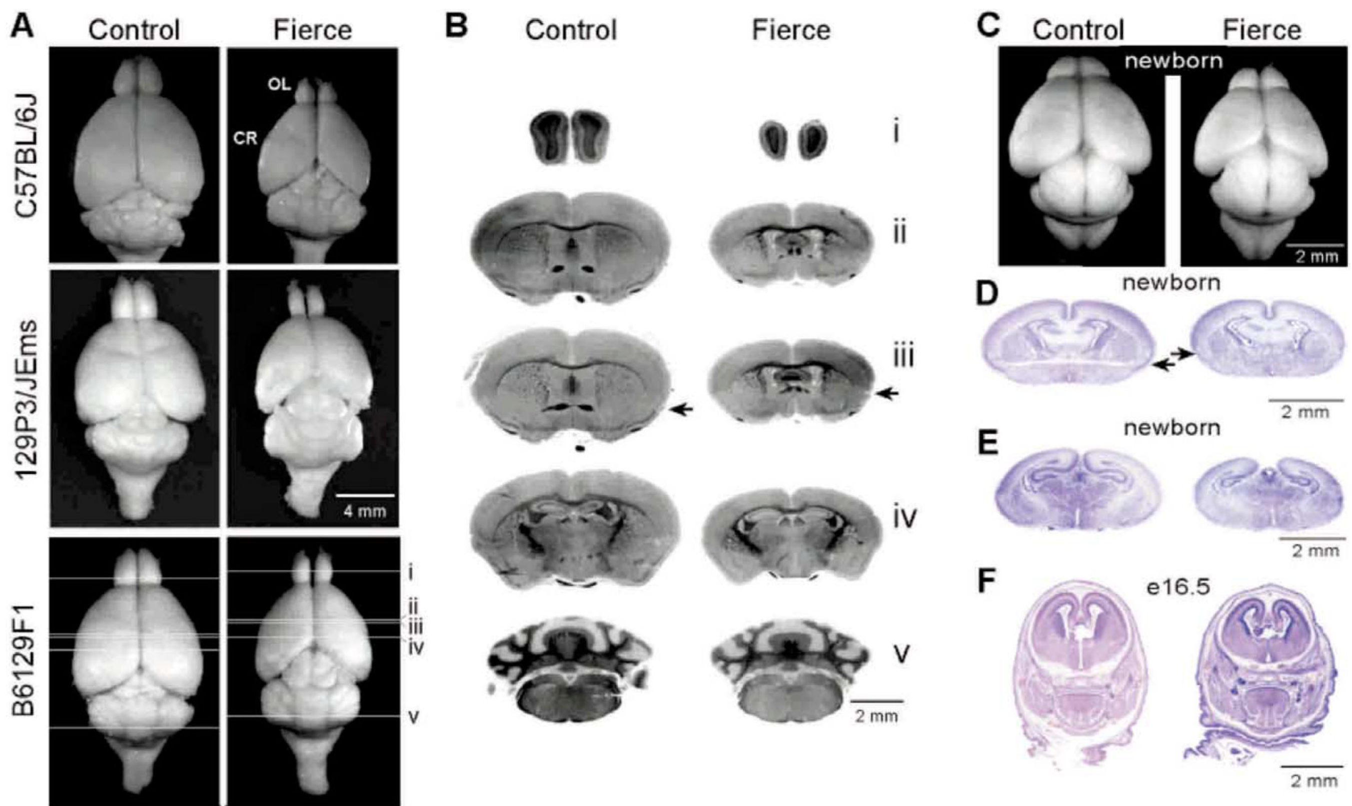

Fig. 3.

Fierce mice have affected brains as adults, newborns and at embryonic day 16.5. (A) Whole brains of *frc* adult mice are differentially smaller in the anterior regions and have hypoplastic cerebrum (CR) and olfactory lobes (OL) on all three backgrounds. (B, D and E) Coronal sections from B6129F1-*frc* mice stained with Luxol fast blue (myelin)/Cresyl violet (neurons) show gross hypoplasia and distortion of the anterior aspects of the brain. The rostral commissure (indicated by an arrow) is malformed; in some animals, it is dramatically thin and wispy as it crosses the midline. (C) Whole brain of newborn B6129F1-*frc* mouse also shows hypoplastic cerebrum and olfactory lobes. (F) Coronal sections from B6129F1 day 16.5 embryos stained with H and E show early evidence of morphological changes.

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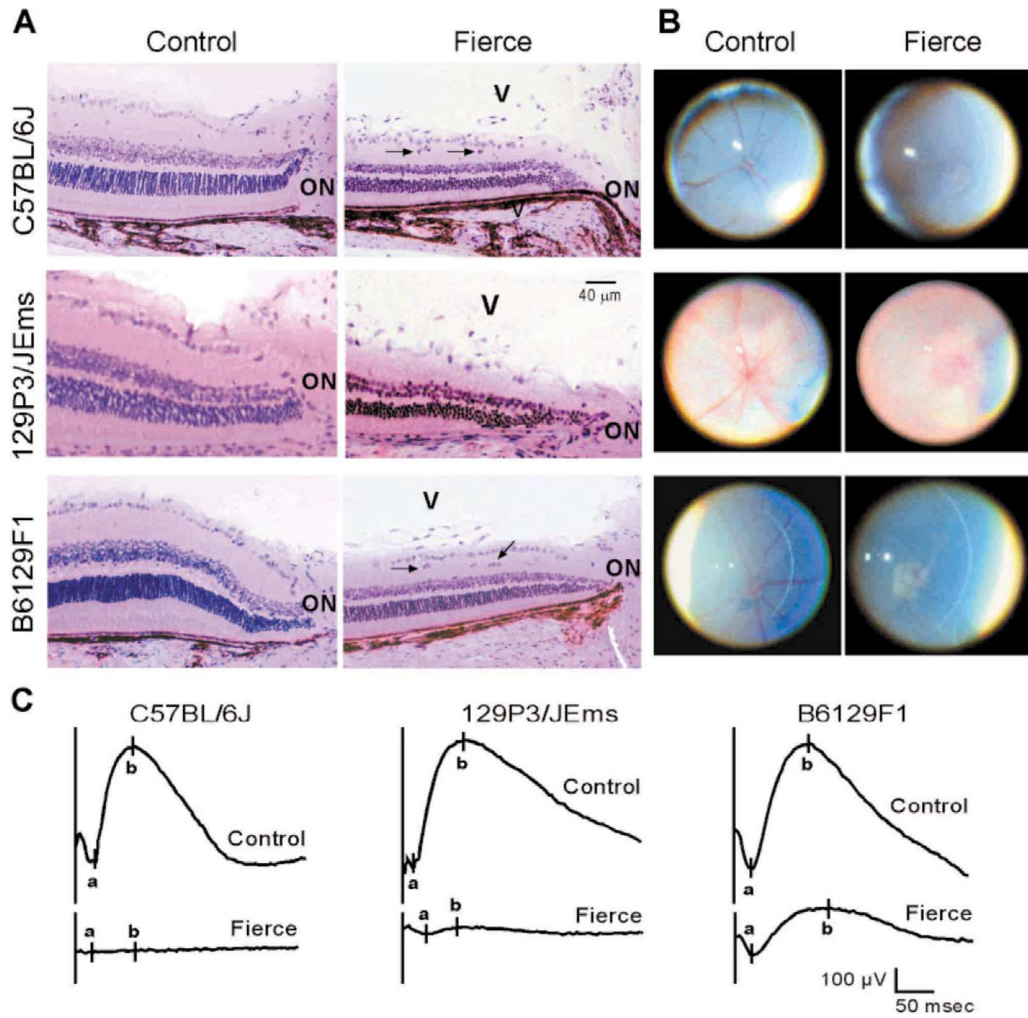

Fig. 4.

Fierce mice have abnormal ocular development. Representative examples are shown from all three backgrounds. (A) Retinas stained with H and E show thinner inner nuclear and photoreceptor layers in 11–12 week old *frc* mice. Arrows indicate displaced retinal ganglion cells. Optic nerves (ON) and inappropriate hyaloid vessels (V) are labeled. (B) Fundus photos show diminished vascularization of the retina in 8-week-old *frc* mice compared to controls. (C) Electroretinograms from 8-week-old mice show impaired maximal response from a 10-μs single flash stimulation (intensity, 73 foot-lamberts-sec) vs. littermate control.

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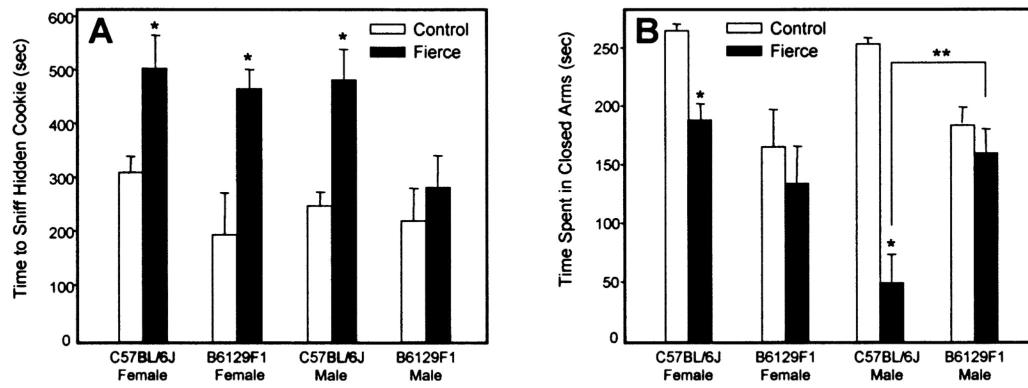

Fig. 5.

Sensorimotor deficits in fierce mice. *, *P*-value for *frc* vs. controls; **, *P*-value for inter-strain comparisons. (A) Time to sniff the hidden cookie. *, *P*<0.005 for male C57BL/6J; *P*<0.008 for female mice. (B) Time spent in closed arms, *, *P*<0.001; **, *P*<0.02.

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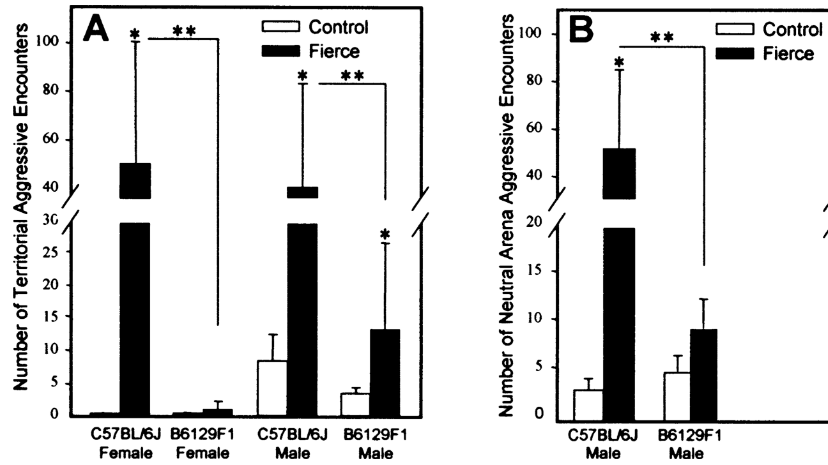

Fig. 6.

Aggressive behaviour in *frc* mice is more prominent in C57BL/6J mice. *, *P*-value for *frc* vs. controls; **, *P*-value for comparisons between backgrounds. (A) Home cage aggressive encounters C57BL/6J: *, $P = 0.02$ for males; *, $P<0.001$ for females; B6129F1: *, $P<0.001$ for males. **, $P<0.002$ for males; **, $P<0.001$ for females. (B) Neutral arena aggressive encounters, *, $P<0.01$; **, $P<0.03$.

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Table 1

Sensorimotor tests reveal differences between fierce and control mice; mean values ± (S.E.M.)

	Genotypes	C57BL/6J male	*P*-value	C57BL/6J female	*P*-value	B6129F1 male	*P*-value	B6129F1 female	*P*-value
Time to sniff hidden cookie (s)	Control	254.7 (25.7)	0.005	316.7 (27.5)	0.006	227.0 (58.9)	0.47	201.2 (77.2)	0.008
	Fierce	488.9 (72.6)		510.2 (56.3)		288.8 (59.4)		472.1 (34.9)	
Time to nibble hidden cookie (s)	Control	463.1 (22.5)	0.1	479.7 (24.0)	0.004	290.6 (65.1)	0.42	225.5 (71.3)	0.002
	Fierce	596.3 (63.6)		600.0 (49.2)		365.6 (63.6)		536.1 (32.1)	
Visual placing	Control	0.72 (0.1)	0.04	0.73 (0.1)	0.33	0.55 (0.3)	1	2.08 (0.3)	0.63
	Fierce	0.22 (0.2)		0.66 (0.1)		0.55 (0.1)		1.75 (0.3)	
Turning in a blind alley (s)	Control	13.9 (1.2)	0.82	12.3 (1.3)	0.003	8.5 (0.9)	0.24	18.8 (8.7)	0.13
	Fierce	13.0 (3.4)		20.3 (2.6)		10.8 (1.7)		17.0 (5.3)	
Turning on an inclined screen (s)	Control	10.6 (1.1)	0.02	7.5 (3.2)	0.14	6.3 (0.8)	0.02	6.5 (0.9)	0.008
	Fierce	37.0 (17.1)		12.2 (3.0)		20.5 (6.4)		18.9 (4.9)	
EPM: number of visits to open arms	Control	0.3 (0.1)	0.007	1.1 (0.3)	0.58	2.2 (0.6)	0.65	1.0 (1.5)	0.1
	Fierce	6.0 (3.1)		0.7 (0.3)		1.8 (0.5)		2.1 (1.2)	
EPM: time in open arms (s)	Control	0.68 (0.03)	0.007	8.9 (4.6)	0.55	19.1 (6.0)	0.12	7.7 (4.1)	0.1
	Fierce	103.0 (55.0)		10.7 (8.7)		65.3 (19.7)		55.4 (40.8)	
EPM: number of visits to closed arms	Control	6.5 (0.6)	0.8	5.8 (0.7)	0.28	8.9 (0.7)	0.001	6.3 (1.1)	1.0
	Fierce	4.0 (2.8)		4.0 (1.2)		4.7 (0.8)		6.4 (1.4)	
EPM: time in closed arms (s)	Control	259.0 (5.0)	0.001	270.0 (4.3)	0.001	188.5 (14.7)	0.33	170.9 (26.9)	0.5
	Fierce	52.0 (22.6)[a]		192.7 (13.7)		164.3 (19.7)[a]		145.5 (27.3)	

EPM, elevated plus maze. Note: comparisons with *P*-values > 0.05 lack sufficient power to exclude the null hypothesis.

[a] $P < 0.02$ for male fierce mice, C57BL/6J versus B6129F1.

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Table 2

Hormonal concentrations did not differ between fierce and control mice; mean values + (S.E.M.)

	Genotype	Sex	C57BL/6J	B6129F1
Testosterone (ng/ml)	Control	F	<0.1	
	Fierce	F	<0.1	
	Control	M	2.80 (0.96)	
	Fierce	M	2.89 (1.63)	
Corticosterone (ng/ml)	Control	F	61.7 (4.4)	190.4 (61.6)
	Fierce	F	72.0 (12.6)	257.9 (28.4)
	Control	M	25.8 (1.7)	89.4 (20.8)
	Fierce	M	47.6 (19.6)	121.8 (19.3)