Motor and Behavioural Abnormalities Associated with Persistent Spontaneous Epilepsy in the fvb/n Mouse Strain

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Summary

The FVB/N mouse strain (*Mus musculus*) is often used for generation of transgenic animals. However, it has also been the object of several studies in epilepsy research due to its increased susceptibility to develop spontaneous and induced seizures and its sensitivity to seizure-triggered neuronal death.

We aimed to analyse behavioural changes observed in FVB/N mice that had seizure episodes throughout life. For this, we assessed the behaviour profile of 52-week old FVB/N animals displaying repeated spontaneous seizures, using the SHIRPA protocol. These epileptic mice also displayed a marked gait ataxia and decreased locomotor and exploratory activity. Moreover, these animals weighed less than control mice, and displayed increased signs of aggression and vocalization behaviours. Analysis of the data by clusters showed that in the epileptic mice there were significant deficits in the SHIRPA "spinocerebellar function", "neuropsychiatric function" and "muscle and lower motor neuron function" scores, suggesting extensive brain damage caused by repeated experience of seizures.

Introduction

The FVB/N mouse strain is one of the most commonly used for the generation of transgenic mouse models, mainly due to its defined inbred background, high reproductive capacity and the large dimensions of pronuclei which facilitate the microinjection of genetic material (*Taketo et al. 1991*). However, with the increasing number of studies that characterized the behaviour, pathology and anatomy of transgenic mouse models, several pieces of evidence started to emerge that raised doubts upon the appropriateness of using this strain for behavioural studies. For instance the FVB/N mouse strain carries the *rd* mutation (for retinal degeneration) leading to visual impairment that was shown to result in a low performance in spatial and non-spatial cognitive tasks (*Royle et al. 1999*; *Voikar et al. 2001*; *Nguyen & Gerlai 2002*; *Pugh et al. 2004*). In addition, a behavioural comparison between the FVB/N and C57Bl/6 strains using four different tests revealed that FVB/N animals display increased anxiety-like behaviour, aggression, hyperactivity and impairment in learning in the Morris water maze paradigm (*Mineur & Crusio 2002*).

The FVB/N strain was also described as being susceptible to epilepsy. In a sample of transgenic and non-transgenic FVB/N mice with a mean age of 5.8 months in which induced or spontaneous generalized seizures had been observed, the brains of affected animals were shown to display neuronal loss and concomitant astrocyte hypertrophy in the cerebral cortex, hippocampus and thalamus (*Goelz et al. 1998*). The pattern of ischemic neuronal necrosis observed was consistent with the type of lesions associated with status epilepticus in humans. In another study, FVB/N mice after administration of kainic acid demonstrated a more severe seizure response and mortality rate (62%) than the 129/

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Ola, BALB/c and C56BL/6 mouse strains (*Royle et al. 1999*). Although the greater susceptibility to develop seizures was not confirmed in two other studies (*Schauwecker et al. 2004*; *McLin & Steward 2006*), the increased vulnerability of the FVB/N strain to seizure-induced brain damage was confirmed histopathologically in all studies by a greater degree of cell loss in the hippocampus (*Royle et al. 1999*; *Schauwecker et al. 2004*) and a synaptophysin immunoreactivity decrease in the CA3 pyramidal cell layer (*Royle et al. 1999*).

The study by McLin and colleagues (2006) further confirmed the susceptibility of FVB/N animals to neuronal damage resulting from kainate-triggered seizures, extending these findings to many brain regions, such as the neocortex, striatum, thalamus, hypothalamus and amygdala. The hippocampal neurons of FVB/N mice were also shown to be more susceptible to cell death as a consequence of pilocarpine-induced seizures (a model for temporal lobe epilepsy); this was paralleled by the severity of the seizures as well as the associated behavioural changes (*Mohajeri et al. 2004*). Taken together these data demonstrate that the FVB/N strain is more sensitive to the brain excitotoxicity and neurodegeneration resulting from substance-induced and spontaneous seizures.

In a genome-wide search for quantitative trait loci (QTLs) affecting individual susceptibility to seizure-induced excitotoxic neuronal damage, Schauwecker *et al*. 2004, assessing the back-cross (N2) progeny of the C57BL/6 and FVB/N inbred mouse strains, identified a locus on the distal part of chromosome 18 as having the strongest and most significant effect upon this susceptibility. Within this 11cm region *GalR1*, *Atp9b*, and *Nfatc1* were identified as interesting candidate genes for the loci affecting kainate-induced cell death.

With the goal of characterizing behavioural changes associated with persistent epilepsy in the mouse, we compared the behaviour of FVB/N mice suffering spontaneous seizures with that of their unaffected littermates using the SHIRPA protocol. Our results demonstrate that FVB/N animals with persistent epilepsy present severe alterations in neurological function, which may result from neuropathlogical damage in several brain regions previously unknown to be implicated in this model.

Materials and Methods

Animals

FVB/N mice were obtained from Eurogentec and bred to create the study group (n = 42). The mice were housed in type II or type III cages by gender (2-7 animals per cage) under a 12:12 h light-dark cycle with food and water available *ad libitum.* The research protocol followed the guidelines of the European Council Directive (86/609/EEC).

As part of a parallel research project (animals were the non-transgenic controls in a study of a transgenic animal model for a neurological disease), animals were periodically observed at different ages by a single individual. At each age, observation was performed for approximately one hour, for five consecutive days.

During these behavioural tests or by occasional observation in their home cages, approximately 17% of animals were observed to undergo seizures; these were included in the current study as the epileptic group (females: n=3; males: n=4) whereas the other animals were included as the control group (females: n=16; males: n=19). The behavioural tests were performed only on animals that did not manifest seizures at that particular moment; a post-ictal recovery time was always allowed.

The convulsion episodes that were observed in these animals were classified as generalised tonic-clonic seizures (Movie – supplemental data). At 52 weeks of age all animals were evaluated using the SHIRPA protocol. Behavioural tests were performed during the light period. After each mouse had finished a test, the material and the equipment was cleaned with a 70% ethanol solution and dried with paper towelling, to avoid interference with the behaviour of other animals.

SHIRPA protocol

We established a protocol for phenotypic

neurobehavioral assessment based on the primary screen of the SHIRPA protocol, which mimics the diagnostic process of general neurological and psychiatric examination in humans (*Rogers et al. 1997*). Each mouse was placed in a viewing jar (15 cm diameter) for 5 min, transferred to an arena (55 x 33 x 18 cm) and then a series of anatomical and neurological measures were determined. Full details of the SHIRPA protocol are available on the site: http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_summary.html

In addition, we included the vertical pole test (*Wallace et al. 1980*), the footprint pattern test (*Carter et al. 1999*) and the counting of rears during the 5 min in the viewing jar as a measure of exploratory/spontaneous activity. The protocol was adjusted in order to minimize animal handling and to generate uniformity in waiting times between tests (*Rafael et al. 2000*).

Vertical pole test

This test was performed on a wooden pole of 2 cm diameter approximately and 40 cm long, wrapped with cloth tape for improved traction. The mouse was placed in the center of the pole, which was held in a horizontal position. Then the pole was gradually lifted to a vertical position. Latency to fall off the pole was recorded with a maximum time of 1 min.

Footprinting pattern test

The footprint test was used to compare the gait of epileptic and control FVB/N mice. To obtain footprints, the hind- and forefeet of the mice were coated with black and red non-toxic paints, respectively. A clean sheet was placed on the floor of the runway for each run. The animals were then allowed to walk along a ramp 100 cm long, and 4.2cm wide (with 10 cm high walls) into an enclosed box. Each animal was allowed to achieve one valid trial. The footprint patterns were analyzed for two step parameters (all measured in centimeters): the front- and hind-base width. For each step parameter, three values were measured for 3 consecutive steps, with the exclusion of the first four steps to allow animal habituation.

Statistical analysis

Behavioural data were subjected to the non-parametric Mann-Whitney U-test when variables were non-continuous or when a continuous variable did not present a normal distribution (Kolmogorov-Smirnov test $p<0.05$). Continuous variables with normal distribution (K-S test $p>0.05$) were analyzed with the Student t-test. When differences were not observed between genders of each group, data from males and females were pooled, and statistical comparisons were performed using the total sample. Statistical analysis of the footprinting test took into account missing data because some animals did not achieve a valid trial. All statistical analyses were performed using SPSS 14.0 and a critical value for significance of $p< 0.05$ was used throughout the study.

Results

SHIRPA protocol

The primary SHIRPA screen provides a comprehensive assessment of basic neurological and physical characteristics of mice (*Rogers et al. 1997*). In this study, it allowed the identification and quantification of significant motor and behavioural abnormalities associated with established spontaneous epilepsy in the FVB/N genetic background.

In general, epileptic animals showed decreased body weight (females $p< 0.01$; males $p=0.019$) (Fig 1A), decreased spontaneous locomotor activity in the arena (number of squares entered by all four feet in 30 seconds) ($p<0.01$) (Fig. 1B), decreased number of rears ($p< 0.01$) (Fig. 1C) and decreased spontaneous activity in the viewing jar ($p<0.01$) (Table 1) and an increased number of urination occurrences ($p< 0.01$) (Fig. 1D).

Affected FVB/N mice also presented a reduced tail ($p<0.01$) and pelvic elevation ($p<0.01$) (Fig. 2B), abnormal gait ($p<0.01$) and body position ($p<0.01$), palpebra closure ($p<0.01$) and piloerection ($p<0.01$). Neurological symptoms, such as limb grasping

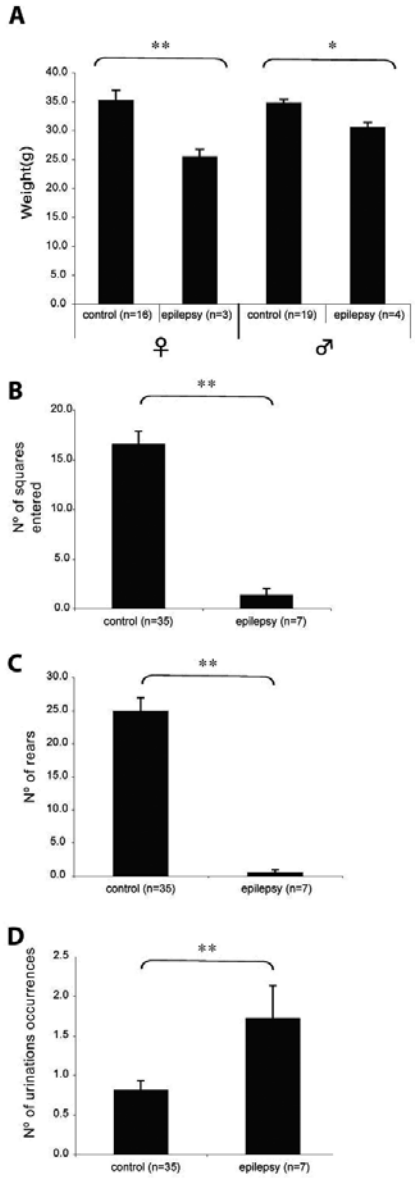

Figure 1. Behavioural data showing differences between epileptic and control FVB/N mice in (A) weight, (B) locomotor activity, (C) number of rears, and (D) urination index. Bars indicate means ± SEM of each group. *Asterisks* indicate statistically significant differences in the performance of normal and epilepsy animals (* p<0. 05; ** p<0. 01).

(p<0.05), limb clasping (p<0.05), tremors (p<0.01) and trunk curling (p<0.01) were more frequent in epileptic FVB/N mice than in the controls (Fig. 2A). These animals also presented a higher hindlimb tonus (p<0.01), a deficit in the negative geotaxis reflex (p<0.01) and in the touch escape response (p<0.01). A decrease in the performance in the wire manoeuvre test was detected in epileptic males only (p< 0.01). Footprinting analysis confirmed that epileptic animals presented marked gait impairments, including a significant enlargement of the frontbase and hindbase width (Fig 2C-E).

Interestingly, increased signs of aggression (p<0.05) and vocalization behaviours (p<0.05) were observed in epileptic animals during the manipulation period in the SHIRPA protocol (Table).

For the other parameters assessed in the SHIRPA protocol we did not find statistical significant differences, except for an increase in time spent holding in the vertical pole test by epileptic females (n=3) when compared to normal females (n=16) (data not shown), the significance of which is unclear to us.

Analysis of the SHIRPA data by clusters (*Rogers et al. 1997*) was performed to evaluate specific functional profiles in control and affected animals. Statistically significant differences (p<0.01) between epileptic and non-epileptic groups were detected for (i) spinocerebellar function; (ii) neuropsychiatric function; and (iii) muscle and lower motor neuron function (Figure 3).

Discussion

The detailed characterization of animal models of seizure susceptibility and reactivity has given an essential contribution to our understanding of the physiological and behavioural changes associated with human epilepsy (*Stables et al. 2002*; *Stables et al. 2003*). Many of these models require induction of epilepsy through administration of a substance such as kainic acid or pilocarpine. The present study provides an analysis of a spontaneous phenotype of seizures in an inbred mouse strain, FVB/N; the

Table 1. Summary of SHIRPA scores that differed between control and epileptic FVB/N animals. Median (quartile ranges) are represented for each group and the p value of Mann-Whitney U-test is shown.

Shirpa scores			
	Control (n=35)	**Epilepsy (n=7)**	**Mann-Whitney U-test**
Body position	4.00 (1.00)	3.00 (1.00)	**p<0,01**
Spontaneous activity	2.00 (3.00)	1.00 (1.00)	**p<0,01**
Tremors	0.00 (1.00)	1.00 (1.00)	**p<0,01**
Palpebra closure	0.00 (1.00)	1.00 (1.00)	**p<0,01**
Piloerection	0.00 (1.00)	1.00 (1.00)	**p<0,01**
Gait	0.00 (2.00)	1.00 (1.00)	**p<0,01**
Pelvic elevation	2.00 (2.00)	1.00 (2.00)	**p<0,01**
Tail elevation	1.00 (1.00)	0.00 (2.00)	**p<0,01**
Touch scape	2.00 (3.00)	1.00 (1.00)	**p<0,01**
Trunk curl	0.00 (1.00)	1.00 (1.00)	**p<0,01**
Limb grasping	0.00 (1.00)	1.00 (1.00)	**p<0,05**
Limb clasping	0.00 (3.00)	3.00 (3.00)	**p<0,05**
Limb tone	2.00 (2.00)	3.00 (1.00)	**p<0,01**
Negative geotaxis	0.00 (4.00)	3.00 (4.00)	**p<0,01**
Agression	0.00 (1.00)	0.00 (1.00)	**p<0,05**
Vocalization	0.00 (1.00)	1.00 (1.00)	**p<0,05**

advantage of this type of study is that it avoids any direct effects of the drugs that may add to the effects of the seizures, thus confounding the analysis in the induced models. Our results demonstrate that animals with persistent epilepsy display important correlated behaviour abnormalities, detectable in several of the tasks included in the SHIRPA protocol.

The main limitation of our approach is that, as seizures in our animals were not continuously recorded, we cannot completely exclude the possibility that the control group also included affected animals. However, the differences obtained for the different behavioural tests were highly statistically significant, suggesting that this hypothetical interference in the control group might be very limited , if present at all..

FVB/N animals with recurrent epilepsy weighed less than control mice at 52 weeks of age. The decreased body weight of epileptic mice and the presence of piloerection might be the result of general morbidity, as previously described in these animals (*Goelz et al. 1998*). In addition, epilepsy-experienced animals presented an increase in the number of urination occurrences (micturition), which has also been described in other animal models of epilepsy (*Midzyanovskaya et al. 2005*) and in human patients (*Nowak et al. 1985*).

The epileptic FVB/N animals also showed an increase of aggression and vocalization during the manipulation period in the SHIRPA protocol. Increased aggressive behaviour has also been described in humans in association with some types of epilepsy (*Haller & Kruk 2006*); this could be explained by dysfunction of aggression-controlling regions such as the downward stimulatory stream that includes the medial amygdala, hypothalamic attack area, periaqueductal grey and locus coeruleus, but also forebrain cortical structures (*Halasz et al. 2002*). Recent studies on mouse

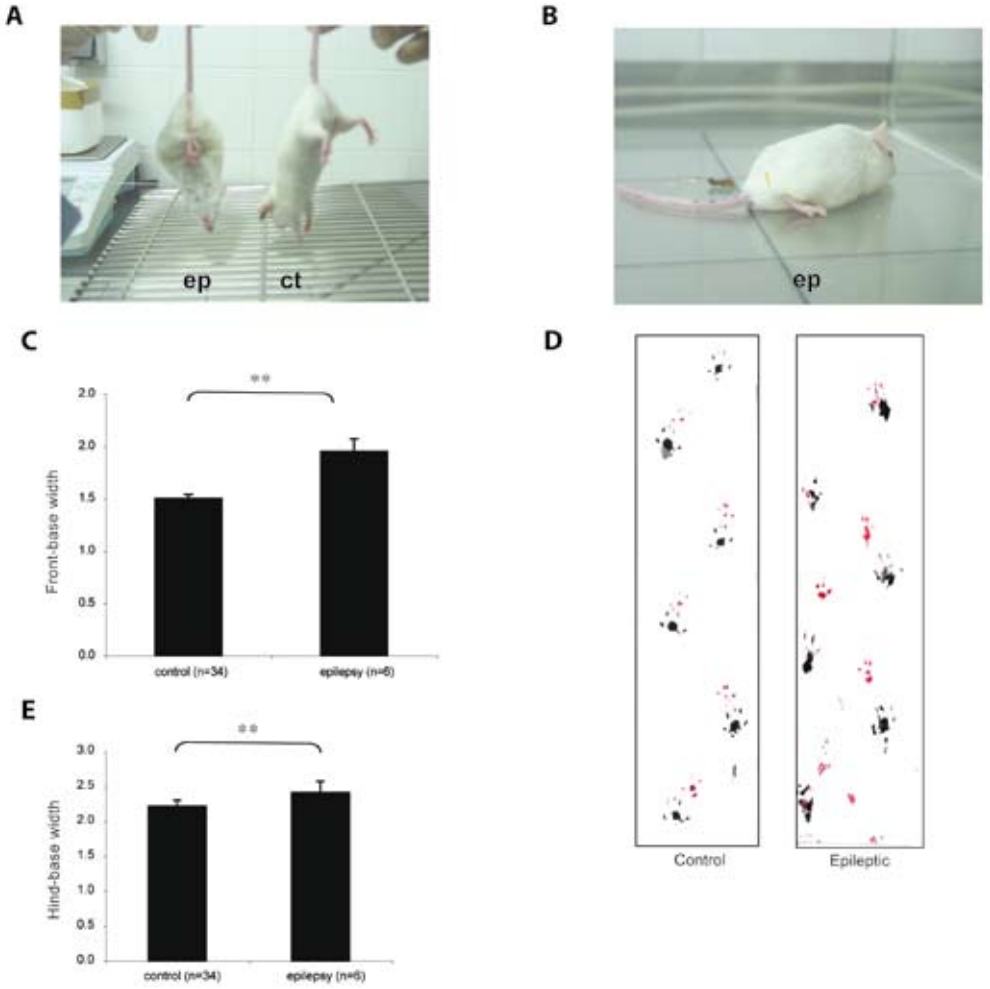

Figure 2. Animal observations during SHIRPA protocol revealed that epileptic FVB/N mice presented neurological symptoms, such as limb grasping, limb clasping and trunk curling (A) and a reduced pelvic elevation (B). Quantitative analysis of the footprint pattern produced by epileptic and control groups at 52 weeks of age: frontbase width (C) and hindbase width (E). Bars indicate means ± SEM of each group. *Asterisks* indicate statistically significant differences in the performance of normal and epilepsy animals (** p<0. 01). (D) Footprinting pattern of control (Ct) and epileptic (Ep) animals.

models of aggression suggest that an imbalance in the activation of the central amygdala and lateral/ventrolateral periaqueductal gray *versus* that of the septum and dorsolateral periaqueductal gray underlie the expression of violent attacks under various circumstances (*Haller et al. 2006*). Important to the context of the present study is the fact that none of these brain regions has been histopathologically characterized in FVB/N mice with spontaneous seizures.

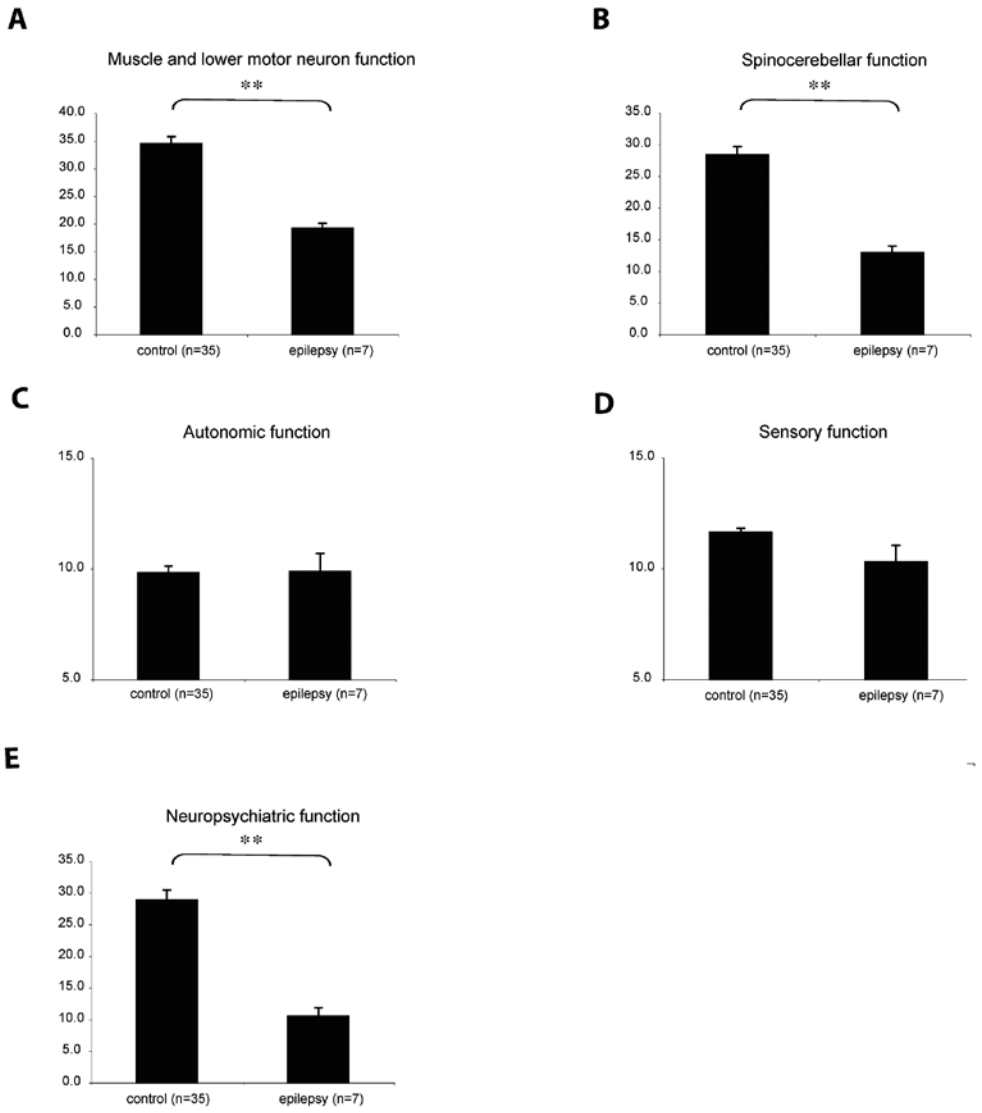

Figure 3. Cluster analysis of scores by specific functions, for epilepsy and control groups at 52 weeks of age. Bars indicate means ± SEM of each group. *Asterisks* indicate statistically significant differences in performance between normal and epileptic animals (** p<0. 01).

Basic activity monitoring has been reported to be useful for distinguishing seizure-prone from control strains mice. For example, in EL mice, a natural model of multifactorial idiopathic epilepsy (*Imaizumi et al. 1959*) that has been validated through electroencephalographic studies, (*Suzuki & Nakamoto 1977*; *Suzuki 1976*), it has been shown that 40-day-old animals prior to the age of seizure

onset (70-90 days) exhibited spontaneous nocturnal locomotor hyperactivity (*Drage & Heinrichs 2005*). In a study with young adult FVB/N mice (12 weeks) with no description of seizure occurrence, this strain was described to exhibit an abnormal circadian behaviour with a fragmented and arrhythmic activity pattern and increased activity during the light phase (*Pugh et al. 2004*). In contrast, our results suggest that FVB/N epilepsy-experienced animals present a deficit in spontaneous activity and exploratory behaviour during the diurnal period, mainly evaluated by the decrease of number of squares in the arena and in the number of rears in the viewing jar. Although we have not performed circadian activity evaluation, our interpretation is that this reduction is probably due to the motor impairment that these mice manifest.

A variety of seizure-prone mutant mice, including ducky, stargazer, "lethargic" and tottering (*Meier 1968*; *Dung & Swigart 1971*; *Meier & MacPike 1971*; *Noebels et al. 1990*), manifest a moderate to severe ataxia (*Fletcher & Frankel 1999*). This is not typically observed in human patients with epilepsy, with the exception of children with Dravet syndrome (or severe myoclonic epilepsy in infancy - SMEI), a severe form of generalized epilepsy with febrile seizures, who at a later stage manifest absence seizures, myoclonic seizures, simple or complex partial seizures and ataxia (*Claes et al. 2001*). Our results show that FVB/N mice with recurrent epilepsy also manifest gait ataxia, evaluated by the significant enlargement of the front-base and hind-base width. Additionally, these epileptic animals present a decrease in muscle strength, higher hindlimb tonus and other neurological symptoms such as limb grasping, limb clasping, tremors and trunk curling, revealing significant deficits both in the spinocerebellar function and in the muscle and lower motor neuron function. Again, this pattern of dysfunction suggests that other brain regions besides the hippocampal mossy fiber system (*Mineur & Crusio 2002*) might be affected in FVB/N mice with spontaneous seizures. Whether dysfunctions in these additional areas in epileptic FVB/N mice are the cause, or rather a consequence of the seizures themselves is still unknown, but certainly a relevant issue to address in future studies.

The knowledge of murine susceptibility genes for seizure-induced cell death could be useful for the rapid identification of key molecular targets for neuroprotective drug design. We propose that the FVB/N mouse strain could be of great importance in the identification of such genes, and for that reason it is important to further characterize the neurological and neuropathological phenotype and the natural history of epilepsy in this mouse strain. Our results suggest that in addition to neuropathological lesions in the cerebral cortex, hippocampus and thalamus previously described in this model, other brain regions must be severely affected in this animal model (*Goelz et al. 1998*), probably as a consequence of seizure experience. The detailed analysis of this animal model could contribute to clarify the manner in which seizure-induced lesions may cause secondary neurological and behavioural alterations in persistent epilepsy.

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