Developmental stability in a cystic fibrosis mouse model

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Summary

The aim of this study was to investigate the influence of cystic fibrosis (CF) and chronic experimental lung infection with *Pseudomonas aeruginosa* on developmental instability and behaviour in the transgenic *Cftr*^{tm1Unc} -TgN(FABPCFTR) mouse compared to different heterozygote ($CFTR^{+/-}$) and wildtype ($CFTR^{+/+}$) controls. Developmental instability measured as fluctuating asymmetry (FA), body weight and open-field behaviour were assessed in $CFTR^{-/-}$, $CFTR^{+/-}$ and $CFTR^{+/+}$ mice. FA and different behavioural tests were investigated in relation to tracheotomy and lung infection with *P. aeruginosa*. Body weight was in general decreased in the $CFTR^{-/-}$ mice and increased in the $CFTR^{+/-}$ mice. $CFTR^{-/-}$ mice had a significantly higher degree of FA (4%-5.5%) than all other groups (1%-3%) ($P<0.001$), while having cystic fibrosis did not seem to influence the behaviour of these mice indicating that the clinical impact from the model is rather low, which is positive from a welfare point of view. FA and motor performance was influenced by neither the lung infection nor the tracheotomy. Tracheotomy increased the level of fear in the light-dark box ($P<0.05$), and the lung infection decreased activity in the open field ($P<0.05$). From this we may conclude that well-being expressed as changed behaviour is a result of the lung infection more than a consequence of the mutation.

Introduction

Several transgenic mouse models (*Colledge et al., 1995; Dickinson et al., 1995; Johansen & Høiby, 1995; Snouwaert et al., 1992; Zhou et al., 1994*) have been developed for the most common autosomal recessive genetic disorder in the Caucasian population (*Dickinson et al., 1995*), cystic fibrosis (CF), which is defective Cl- transportation over the mucosal surfaces (*Knowles et al., 1983*). CF mice, generated by a targeted mutation of the CFTR-gene combined with the insertion of the human mutation by microinjection (*Kraemer et al., 1998*), are commonly applied as models of pulmonary infection with Pseudomonas aeruginosa (*Kent et al., 1997; Stotland et al., 2000; Van Heeckeren & Schluchter, 2002*). CF influences growth (*Hausler et al., 1994; Lai et al., 1998*), bone mineral density (*Haworth et al., 1999*), and enamel development (*Wright et al., 1996*), which may affect the developmental stability of the individual. Chronic pulmonary infection with P. aeruginosa may affect developmental stability of the individual, as growth is also affected by pulmonary dysfunction in CF patients (*Zemel et al., 2000*). Heterozygotic mice may be affected as well, as they in some aspects differ from healthy control mice, e.g. in the development of asthma (*Dahl, 1998; Schroeder et al., 1995*). Such developmental instability may be measured as fluctuating asymmetry (FA), which reflects small, random deviations from symmetry in otherwise bilaterally symmetrical traits (*McKenzie & O´Farrel, 1993; Parsons, 1992; Wilson & Manning,*

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1996). Other heritable diseases, e.g. Down´s syndrome, are found to increase FA in humans (*Townsend, 1983*). Activity and other behavioural measures in the open field are used to evaluate the well-being of laboratory rodents (*Eskola & Kaliste-Korhonen, 1998*); and in humans, physical activity have been used as a parameter to determine the influence of CF on daily life and thereby the general well-being of the individual (*Britto et al., 2000*). The aim of this study was to study the influence of CF and chronic lung infection with P. aeruginosa on developmental stability and behaviour in the transgenic $Cftr^{tm1Unc}$ -TgN(FABPCFTR) mouse.

Materials and Methods

This type of study was approved by the Danish Animal Experimentation Board. $Cftr^{tm1Unc}$-TgN (FABPCFTR)/Jax mice (Jackson Laboratory, Maine, USA) originating from the strains 129 and C57BL (donor strain: 129P2 via E14TG2a ES cell line, into a C57BL blastocyst (Snouwart et al., 1992)) were inbred as a new strain, F?+3 on arrival. Health monitoring according to FELASA guidelines (*Nicklas et al., 2002*) revealed no mandatory agents. Mice were housed with lights on from 9 pm to 9 am (pulmonary infection experiment) or from 6 am to 6 pm (all other experiments) at 20-22°(C, 55-80% relative humidity, and ten to fifteen air changes per hour in Macrolon Type III cages with bedding and environmental enrichment (Tapvei, Vaikkojoentie 33 Fin-73620, Kortteinen). They were fed Altromin 1324 (Altromin Denmark, Chr. Petersen A/S, DK-4100 Ringsted, Denmark) and water *ad libitum* with Gentamicin (0.5 mg/l), (Garamycin® 2 mg/ml, Schering-Plough A/S, Farum, Denmark).

Homozygotic $CFTR^{-/-}$ ($Cftr^{tm1Unc}$ -TgN(FABPCFTR) mice were compared in their developmental stability, growth and behaviour with wild type and heterozygotic control mice, i.e. both background strains (129s6/Sv/Bom and C57BL/6J/Bom (Taconic M&B (Laven, Denmark)), and F1 and F2 generations of these and $CFTR^{+/-}$ on different genetic combinations (Table 1) at eight weeks of age. For tracheotomy and chronic lung infection with *P. aeruginosa*, two groups of five eight-weeks-old male $Cftr^{tm1Unc}$ -TgN(FABPCFTR) mice anesthetized with fentanyl, fluanisone and midazolam (Hypnorm® Janssen-Cilag; Dormicum® 5 mg/ml, Roche) as previously described (*Flecknell, 1996*), were inoculated in the left lung with $5x10^7$ CFU/ml of *P. aeruginosa* 57388 (a mucoid clinical CF isolate (*Hoffmann et al., 2002*)) in 0.04 ml 0.9% sterile NaCl or with pure saline (*Johansen, 1996*). 0.001 mg buprenorphine (Anorphin, Gea, Frederiksberg, Denmark) and 1 ml 0.9% NaCl was given s.c. 6, 18, 30 and 42 h (NaCl only) after the operation. Body weight and FA according to Figure 1 was measured before, three and seven days after the

Table 1

Abbreviation	Father	Mother	Genotype, pups N (males + females)	
129s6/Sv	129s6/Sv	129s6/Sv	CFTR+/+	8 + 16
C57Bl/6J	C57Bl/6J	C57Bl/6J	CFTR+/+	10 + 13
F1	129s6/Sv or C57Bl/6J	129s6/Sv or C57Bl/6J	CFTR+/+	19 + 15
F2	F1	F1	CFTR+/+	14 + 14
129CF	CFTR-/-	129s6/Sv	CFTR+/-	13 + 11
C57CF	CFTR-/-	C57Bl/6J	CFTR+/-	13 + 9
F1CF	CFTR-/-	F1	CFTR+/-	13 + 18
CF	CFTR-/-	CFTR-/-	CFTR-/-	13 + 11

Breeding, genotype, abbreviations and number of animals used in the experiment for evaluating the effect of the CFTR mutation

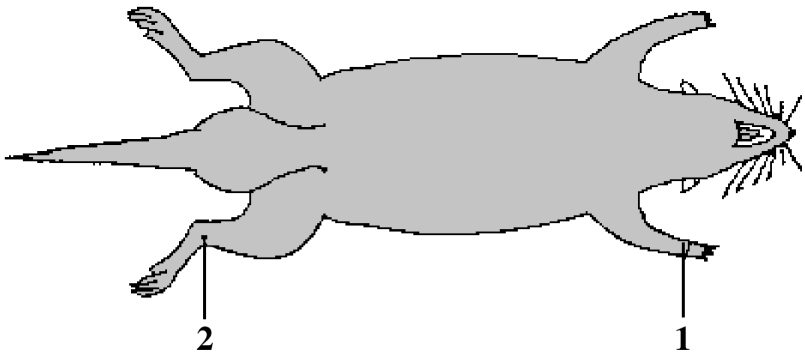

Figure 1. Fluctuating asymmetry (FA) was measured using a digital caliber with constant pressure to the nearest 0.01 mm (Mitutoyo, Mitutoyo Corporation, Japan). Traits used were 1) the width of carpal bones and 2) the width of the joint between tibia and tarsal bones. Each site is measured twice. Trait size was determined by calculating the mean of the left and right traits. Absolute FA was defined as right-minus-left trait size. Relative FA of a trait was defined as absolute FA divided by trait size (relative FA=absolute FA/($^1/_2$*size of right side + $^1/_2$ * size of left side)). Mean relative asymmetry was the mean relative asymmetry of the two different traits using the mean of the numeric values of the relative FA of the individual traits. The absolute asymmetry (not the numeric value) of each trait was used for examining the type of asymmetry. The width of carpal bones (outside the animal) (site 1) and the width of the joint between tibia and tarsal bones (outside the animal) (site 2) were measured. These traits were in earlier studies found to express FA in mice and rats (*Stub et al., 2002*).

operation. Motor performance was tested as a footprint test before and two days after the operation. Anxiety was tested in the light-dark box two days after the operation and compared with age-matched controls. Open-field behaviour was tested three days after the operation and compared with age-matched controls. In the open field, latency to move, locomotor activity and vertical activity were evaluated in a circular open field arena, 90 cm in diameter and 40 cm in height divided by twelve lines 30° apart radinating from a central 30 cm circle. The mouse was initially placed and then recorded by video without humans or other mice in the room for ten minutes during the light period. Between sessions the test arena was cleaned. Anxiety-related behaviour was tested in a light-dark box using a Macrolon Type III cage divided into two equally sized compartments connected by a 6 cm x 6 cm opening, one with white walls and normal daylight and one closed with black walls and top, in which the mouse was placed. The latency to enter the light compartment, the number of visits made and the total time spent in the light compartment were observed on video for five minutes without humans and other mice during the dark period. Between sessions, the test arena was cleaned. Motor performance was analyzed by footprint analysis. A piece of preformed paper was placed in an open cage (15 x 50 cm) with the far end of the cage covered in darkness. Mice with red ink on their front paws and blue ink on their rear paws were placed in the open end of the cage leaving prints of the footsteps on the paper when escaping towards the covered area. Steps from walking were analyzed by measuring the length of the steps.

All statistics were made in MINITAB 12.1 (Minitab Inc.). Weight data, which were normally distributed, were analysed by ANOVA to determine the effect of genotype and sex. Relative FA, which did not follow a normal distribution, was

analysed by Kruskal-Wallis test. As absolute FA must follow a normal distribution and have a mean of zero, this was tested by using the Kolmogorov-Smirnov test for normality ($\alpha<0.05$), and ANOVA for difference from zero. Behavioural data were tested for normality using the Kolmogorov-Smirnov test for normality ($\alpha<0.05$), and subsequently tested by the Kruskal-Wallis test. Repeated measures before and after the lung infection were analysed by paired t-test (weight data) or by Wilcoxon Signed Rank Test.

Results

Influence of the mutation

Both the width of carpal bones (Figure 1, site 1) and the width of the joint between tibia and tarsal bones (Figure 1, site 2) satisfied the demands of FA, i.e. the data were normally distributed as shown by the Kolmogorv-Smirnov test and had a mean of zero. No significant differences in body weight were shown between the different strains of mice (Figure 2). Homozygote CFTR$^{-/-}$mice had significantly higher degree of FA (4%-5.5%) than all other groups (1%-3%) ($P<0.001$). Heterozygote mice

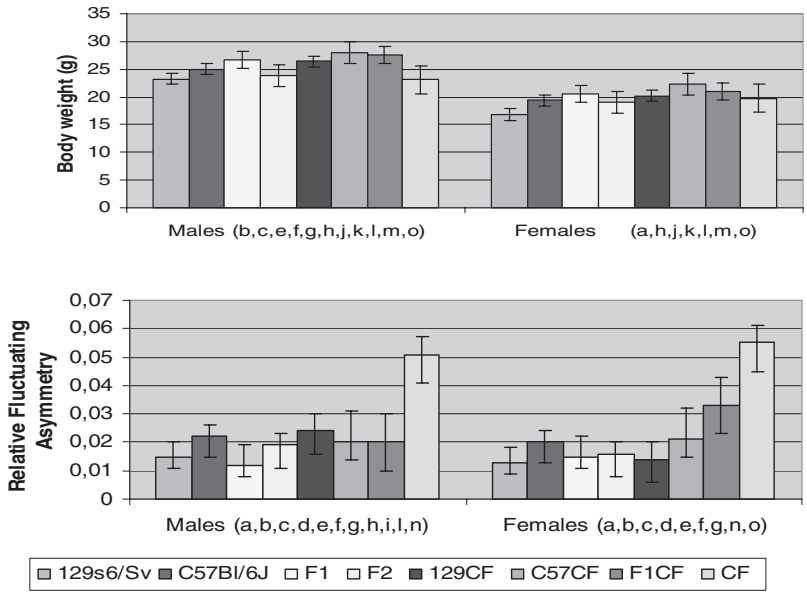

Figure 2. Means of body weight (+/- SD) and medians of relative fluctuating asymmetry (FA) (with 25% and 75% percentils) in 8 week old CFTR-/- mice compared to different heterozygote and wild type controls. Abbreviations are shown in Table 1. Significant differences between: a) CF and 129s6/Sv ($P<0.001$, except in body weight, $P<0.05$), b) CF and C57Bl/6J ($P<0.001$), c) CF and F1 ($P<0.001$), d) CF and F2 ($P<0.001$), e) CF and 129CF ($P<0.001$), f) CF and C57CF ($P<0.001$), g) CF and F1CF ($P<0.001$), h) 129CF and 129 ($P<0.001$ except in FA, $P<0.05$), i) 129CF and F1 ($P<0.001$), j) 129CF and F2 ($P<0.05$ in body weight and $P<0.001$ in VA), k) C57CF and C57Bl/6J ($P<0.001$), l) C57CF and F1 ($P<0.05$ in body weight, $P<0.01$ in FA), m) C57CF and F2 ($P<0.001$), n) F1CF and F1 ($P<0.01$) and o) F1CF and F2 ($P<0.001$ in body weight and $P<0.01$ in FA). So a-d are giving the difference between CFTR-/- and CFTR+/+ ($P<0.001$), e-g the difference between CFTR-/- and CFTR+/- ($P<0.001$) and f-o the difference between CFTR+/+ and CFTR+/- ($P<0.05$).

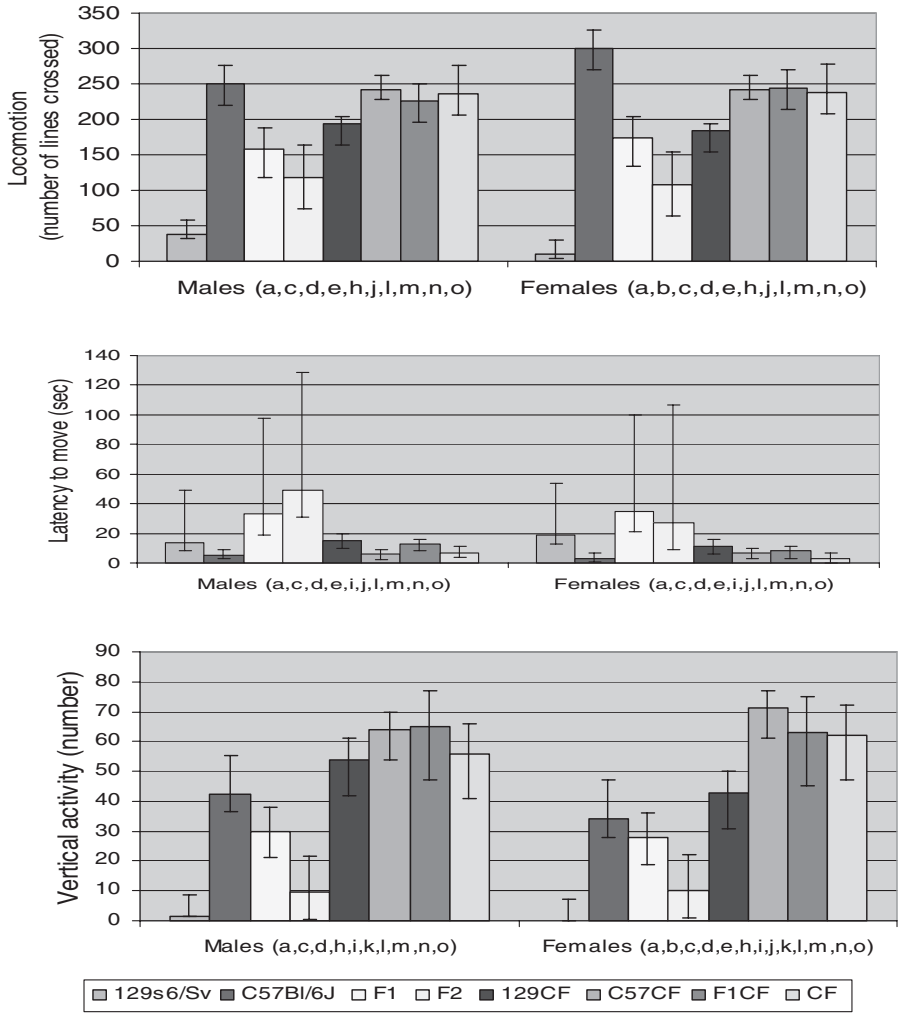

Figure 3. Medians (with 25% and 75% percenties) of locomotion, vertical activity and latency to move in the open field in 8 week old cystic fibrosis mice compared to different heterozygotic and wild type controls. Significant differences between: a) CF and 129s6/Sv ($P<0.001$), b) CF and C57Bl/6J ($P<0.05$), c) CF and F1 ($P<0.001$), d) CF and F2 ($P<0.001$), e) CF and 129CF ($P<0.05$), h) 129CF and 129 ($P<0.001$), i) 129CF and F1 ($P<0.001$), j) 129CF and F2 ($P<0.001$), k) C57CF and C57Bl/6J ($P<0.001$), l) C57CF and F1 ($P<0.001$), m) C57CF and F2 ($P<0.001$), n) F1CF and F1 ($P<0.001$) and o) F1CF and F2 ($P<0.001$). So a-d are giving the difference between CFTR-/- and CFTR+/+ ($P<0.05$), e-g the difference between CFTR-/- and CFTR+/- ($P<0.05$) and f-o the difference between CFTR+/+ and CFTR+/- ($P<0.05$).

from F1CF had a higher degree of FA (2%-3%) than the F1 and F2 generations (1%-1.5%) ($P<0.01$) (Figure 2). There were no sex differences in FA. Significant strain differences were observed in the open field (Figure 3), but CFTR did not seem to be the influencing factor. Sex did not influence behaviour. There were no differences in open field behaviour between heterozygote and homozygote $CFTR^{-/-}$ mice.

Influence of pulmonary infection

In the tracheotomy/pulmonary infection experiment with *P. aeruginosa*, body weight of the mice in the saline group was significantly decreased three days post surgery ($P<0.001$), while this was not the case in the infected group. The mice of the saline group had normalized their weight after seven days (data not shown). FA and motor performance was not influenced by either pulmonary infection or surgery (Figure 4). Both infected and saline-treated mice had fewer visits into the light area in the light-dark box compared to non-operated controls (Figure 5, $P<0.05$), indicating increased anxiety. In the open field, the infected and the saline-treated mice had to move than control mice (Figure 5, $P<0.05$). Locomotion as well as vertical activity in the open field was decreased in the bacterially infected mice compared to saline treated and non-operated controls (Figure 5, $P<0.05$). After the experiment, the lungs were investigated for infection; and as expected, infection was found in the bacterially infected mice, whereas controls were free (data not shown).

Discussion

The most important outcome of this study was that developmental stability expressed as fluctuating asymmetry was significantly decreased in the $CFTR^{-/-}$ mice compared to all heterozygote and wild

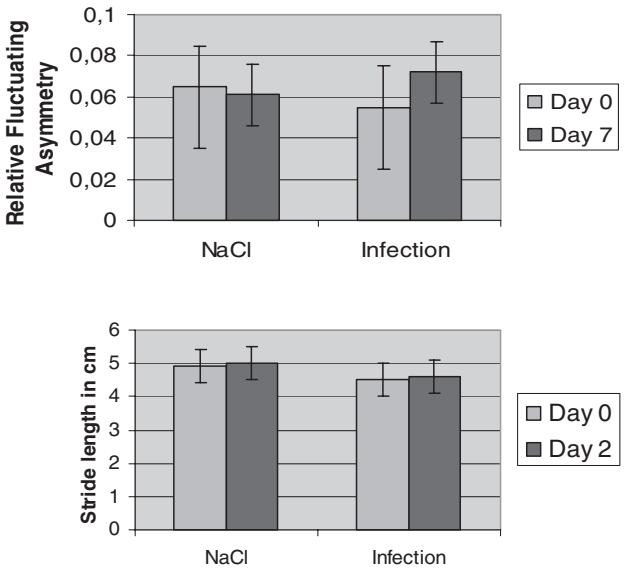

Figure 4. Relative fluctuating asymmetry and stride length (medians with 25% and 75% percentils) before and after the tracheotomy operation with P. aeruginosa bacterieas or 0.9% NaCl (control). No significant differences were seen.

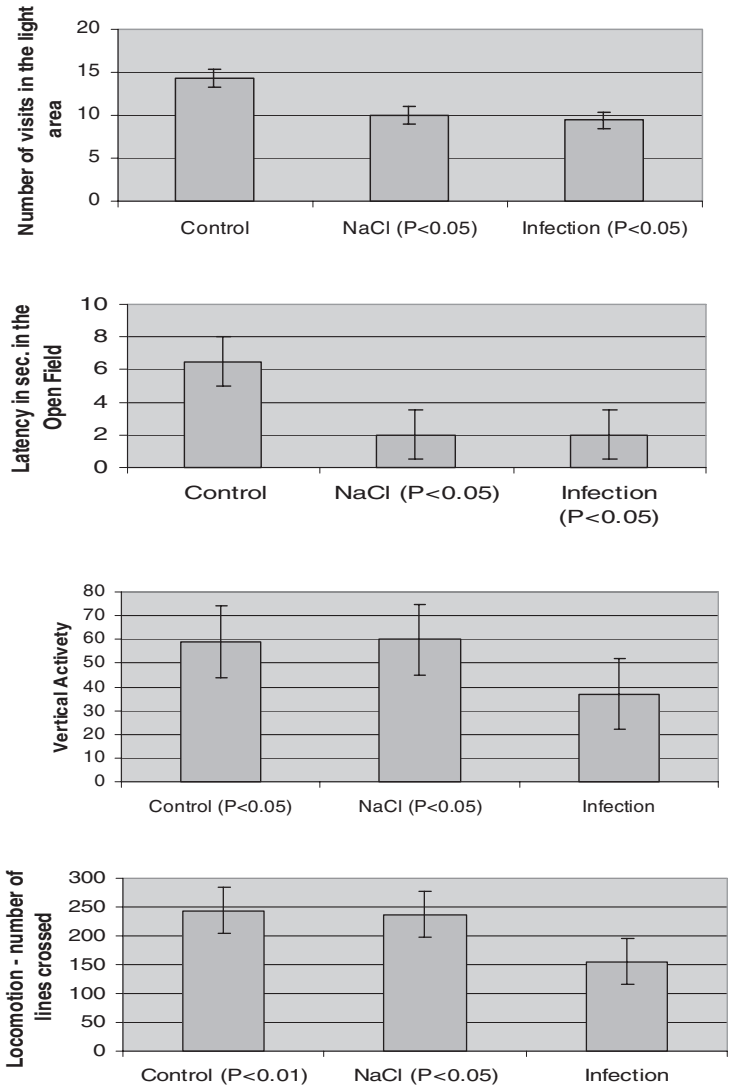

Figure 5. Behaviour in the light-dark box and open field after the challenge with P. aeruginosa or 0.9% NaCl, compared to non-operated cystic fibrosis mice controls (medians with 25% and 75% percentils). In the light-dark box and in latency to move in the open field, animals operated on with NaCl as well as P. aeruginosa bacteria differed significantly from the non-operated controls ($P < 0.05$). In locomotion and vertical activity (number of times, the mouse is raising up at its hind legs) in the open field, animals infected with P. aeruginosa differed significantly from the NaCl- operated as well as the non-operated controls ($P<0.05$).

type control mice. This increased developmental instability in mice with cystic fibrosis may be analogous to those growth abnormalities found in humans with cystic fibrosis (*Hausler et al., 1994; Lai et al., 1998; Wright et al., 1996*). It may, therefore, serve as a model, and may be used in various ways as a parameter in projects involving these mice. It may be argued that the genetic background and not only the lack of CFTR expression may be of importance for this, as this transfection has not been backcrossed on a defined inbred background strain, such as C57BL, and therefore is difficult to compare without a genetically originating bias. However, reduced developmental stability was not found in the heterozygote mice compared to the other strains monitored, and furthermore the difference between $CFTR^{-/-}$ mice and all other strains of mice was rather large. Having cystic fibrosis did not seem to influence the behaviour of these mice indicating that the clinical impact from the model is rather low, which is positive from a welfare point of view. It should be mentioned, that gentamycin acts locally in the intestine and is not absorbed, so influence on CNS and behaviour is not expected and therefore this treatment is of no importance for these observations. The tracheotomy was found to increase anxiety of the animal, but it did not change activity level, whereas the pulmonary infection was found to decrease activity but not change the level of anxiety. The changed activity level was not a result of decreased motor performance, as this was found unchanged by tracheotomy or infection with *P. aeruginosa*. After seven days developmental stability was not influenced by any of these procedures. The reduced activity in the *P. aeruginosa* infected mice is comparable with the reduced exercise capacity found in patients with reduced lung function (*King & Cotes, 1989*) and may in both cases indicate reduced well-being. From this we may conclude that well-being expressed as changed behaviour is a result of the lung infection more than a consequence of the mutation, which is what would have been expected.

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