Impact of IVC housing on emotionality and fear learning in male C3H and B6J mice

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Abstract

Housing conditions are known to influence laboratory animal behaviour. However, it is not known whether housing mice in individually ventilated cages (IVCs) to maintain optimal hygienic conditions alters behavioural baselines established in conventional housing. This issue is important regarding comparability and reproducibility of data. Therefore, we investigated the impact of IVC housing on emotionality and fear learning in male C3HeB/FeJ (C3H) and C57BL/6J (B6J) mice housed singly either in conventional type II cages with wire bar lids (Conventional), or in IVCs of the same size, but with smooth, untextured lids (IVC classic), thus acoustically attenuated from external stimuli and with limited climbing facilities as compared to Conventional. To evaluate the role of climbing, additional mice were kept in IVCs with lids having wire bars ("grid") added to the surface (IVC grid). Spontaneous behaviour, sensorimotor behaviour and fear learning were measured. IVC housing reduced activity and enhanced anxiety-related behaviour in both strains, whereas grooming latency was reduced in B6J only. IVC housing increased Acoustic Startle response in C3H, not in B6J mice. The "grid" did not compensate for these IVC housing effects. In contrast, B6J mice in IVC grid performed best in Fear Potentiated Startle, B6J mice in IVC classic performed worst, suggesting climbing facilities combined with IVC housing facilitate FPS performance in singly housed B6J males. Our data show that IVC housing can affect behavioural performance, and can modulate behavioural parameters in a general as well as a strain-specific manner, both relevant for mouse functional genomics.

Introduction

With the completion of the genome sequences of human and several other species, one of the major challenges in biomedical science is the determination of gene and protein function. The mouse is the most used model organism in the endeavor to develop a complete functional annotation of the human genome and to employ this information to better understand human disease and its underlying physiological and pathological basis (Nadeau et al. 2001; O'Brien and Woychik 2003; Nobrega and Pennacchio 2004). As a consequence, there is an increasing demand for phenotyping facilities for the analysis of mouse mutants (Auwerx et al. 2004). Mouse functional genomics require standardization of mouse handling and housing conditions because minor changes in procedures have been shown to profoundly affect biological variables, thereby challenging the reproducibility of mouse phenotypic data (Champy et al. 2004).

Recently, a mouse phenotyping facility, the German Mouse Clinic (GMC) was established as an open access phenotyping platform (Gailus-Durner et al. 2005) into which mice with varying health status are imported. Thus, to function efficiently, maintenance of optimal hygienic conditions and prevention of the spread of infectious agents are mandatory. To this end, the use of individually ventilated cage (IVC) systems is currently the method of choice.

Although it is known that physiological and behavioural parameters in mice are affected by environmental factors (Terranova et al. 1993; Crabbe et al. 1999; however see Wolfer et al. 2004), unfortunately, details of housing conditions of experimental animals, which may account for differences in results between laboratories (Wahlsten 2001) are still not regularly reported. Since the use of IVCs is relatively recent, but may become the method of choice in the growing number of large scale phenotyping facilities, it is important to assess whether IVC housing alters behavioural baselines established in mice housed in conventional cages.

So far, the effects of IVC housing were only analysed with respect to breeding performance, health status due to cage ventilation rate and cage change frequencies, maintenance of gas concentrations and exposure to aeroallergens (Hoglund and Renstrom 2001; Reeb-Whitaker et al. 2001; Renstrom et al. 2001; Krohn and Hansen 2002; Tsai et al. 2003). Therefore, this study was aimed at comparing the effects of conventional and IVC housing on behaviour of mice of the two inbred strains C3HeB/FeJ and C57BL/6J, since these strains are used for mutagenesis studies (Hrabe de Angelis et al. 2000; Nolan et al. 2000; Banbury Conference, 1997). Due to their solid stainless steel lids, IVCs used in the GMC, firstly, prevent mice to a greater extent from external environmental stimuli than conventional cages, and, secondly, because the IVC lids are smooth and untextured except for the food hopper, they offer mice

less climbing facilities in comparison to conventional cages, which are equipped with wire bar lids. To test whether putative differences in test results could be due to different climbing opportunities, we modified IVC lids by adding custom-made rectangular wire bars ("grid") to their inner surface.

Here we present data for both strains housed either in Conventional, IVC grid or IVC classic conditions and tested for spontaneous behaviour, sensorimotor function and fear learning. The modified Hole Board test (Ohl et al. 2001a; Ohl et al. 2001b; Ohl et al. 2001c) was chosen to assess spontaneous behaviour, because it allows the comprehensive analysis of a range of behavioural parameters indicative of behavioural dimensions such as locomotor activity, exploratory behaviour, arousal, emotionality, object recognition memory and social affinity in a single short test. It was specifically developed by Ohl and coworkers as high-throughput basic behavioural phenotyping screen for laboratory rodents, and is used in a modified version adapted to hygienic and workflow requirements as primary screen in the behavioural phenotyping module of the GMC.

The Acoustic Startle Reflex (ASR) was chosen to assess sensorimotor behaviour, because it is effective in elucidating genetic effects on neural mechanisms of startle behaviour (Davis 1980; Crawley 1999; Plappert and Pilz 2001). The Fear Potentiated Startle (FPS) paradigm was used to assess fear learning, since it is a short and automated test for cognitive function that has been validated for mice (Falls et al. 1997; Falls 2002).

Our data indicate that IVC housing can markedly affect behavioural phenotyping results. Depending on the behavioural parameters measured, we observed general IVC housing effects as well as strain-specific ones when compared to Conventional housing. The "grid" did not compensate for most of these effects. These findings bear relevance for data reproducibility as well as for comparison and interpretation of data across phenotyping facilities which use different caging systems.

Material and Methods

Mice and Husbandry

Two strains of inbred mice, C3HeB/FeJ (C3H) and C57BL/6J (B6), were bred and kept at the German Mouse Clinic. Mice of were born in individual ventilated cages as delivered by the manufacturer (IVC, VentiRacks™, BioZone, Margate, UK). At the age of 6 to 8 days mice were transferred with their mothers to different designed cages (Fig. 1) and weaned at 3 weeks of age. After weaning, male mice from each strain were kept singly in three types of cages (n = 10) as shown in Fig. 1: a) in conventional type II Macrolon® cages with wire bar lids (Conventional) and in IVCs of the same size either b) with solid stainless steel lids as provided by the manufacturer, but with additional wire bars ("grid") fixed to the inner surface (IVC grid) or c) in IVCs as provided by the manufacturer (IVC classic). Mice were kept in a barrier unit at a temperature of 22 to 24 ^{o} C, humidity of 50 to 60 %, 20 air exchanges per hour and a 12/12-hour light/dark cycle. Wood shavings (Altromin, Lage, Germany) and a paper tissue (20 cm x 21 cm, KIMWIPES® Lite 200) were provided as bedding. Mice were fed a standardized mouse diet (1314, Altromin) and provided drinking water (0,2 µl filtered tab water) *ad libitum*. They were tested for microorganisms (Brielmeier et al. 2002) according to the FELASA Recommendations to the level required for re-derived mice by blood collection from the tail vein at the age of 22 weeks (Kraft et al. 1994; Nicklas et al. 2002). All animal experiments were approved by the animal welfare and use committee of the local governmental body.

Experimental design

At the age of 9 weeks, mice were tested in the modified Hole Board (mHB). Two to three days later, Acoustic Startle Reflex (ASR) was measured. Due to technical problems this data could not be analysed and had to be discarded. At the age of 10 weeks Fear Potentiated Startle (FPS) was assessed. At the age of 14 weeks mice were tested again for ASR. To assess a potential impact of age-related hearing loss (Henry and Chole 1980; Zheng et al. 1999) on acoustic startle response, mice were retested for ASR at the age of 20 weeks. All experiments were carried out during the light period of the light/dark cycle.

Modified Hole Board (mHB)

The mHB experiments were carried out between 9:00h and 13:00h. The mHB apparatus was built and the test was carried out according to a modification of the procedures previously

described by Ohl and co-workers (2001b): Different from the original design, in our study, instead of food, for each trial an unfamiliar (a blue plastic tube lid, diameter 2 cm, height 1 cm) and a copy of a familiar object (metal cube, diameter 2 cm, height 1.5 cm; remaining for 48 h in the home cage, removed 24 h before testing) were placed into the same corner of the arena with a distance of 2 cm. Each animal was placed at the start position in the same corner diametrical to the corner where the two objects were placed, facing the board diagonally and was tested for 5 min in moderate light conditions (150 lux in the corners to 200 lux in the middle of the test arena). After each trial, the arena was cleaned and disinfected. All trials were videotaped and tracked by Ethovision 2.3 (Noldus, Wageningen, NL) for total distance travelled and mean velocity calculation. The movement detection threshold was set at a shift of the center of gravity of the animal for at least 1 cm in horizontal direction. A hand-held computer was used by a trained observer to assess line crossings, board entries, rearings on board, rearings in the box, hole exploration, hole visit, familiar and unfamiliar object exploration, immobility, stretched attends, defecation and grooming. Data were analysed by use of the Observer software 4.1 (Noldus, Wageningen, NL) with respect to frequency, latency of first occurrence and duration in % of total observation time. In the result section, only those parameters are shown which were influenced by the housing conditions including locomotion, anxiety, exploration and arousal related parameters.

Startle Apparatus

Acoustic Startle Reflex (ASR) and Fear Potentiated Startle (FPS) were assessed using the startle apparatus and software ("Startle Reflex" for ASR, "Advanced Startle" for FPS) from Med Associates Inc. (VT, USA, Startle Stimulus Package PHM-255A, ANL-925C Amplifier). For FPS, foot shocks were delivered by Stand Alone Shockers (ENV-414s-SR), shock intensity was calibrated with the PHM-265 Shock Current Test Package.

Fear Potentiated Startle (FPS)

In the FPS paradigm conditioned fear is defined as elevated startle response to the conditioned stimulus (CS) after conditioning in comparison to the response to the CS before conditioning.
FPS experiments were carried out between 8:30h and 14:00h. The FPS protocol consisted of three sessions: pre-conditioning, conditioning and post-conditioning, with a 24 h delay between sessions which were carried out according to the protocol developed by Falls (Falls 2002). We modified the pre-conditioning protocol by using startle stimuli intensities (STL) of 95, 100 and 105 dB and a CS of 12 kHz at 60 dB. For conditioning, we used a 0.5 sec foot

shock of 0.4 mA and a variable inter trail interval (180-330 sec). Mean startle amplitudes were calculated over all three startle stimulus intensities, response to CS was calculated as percentage: % response to CS = [(CS - STL)/STL]*100.

Protocol and data calculation for post-conditioning were exactly the same as for pre-conditioning. Fear potentiation was defined as a significant increase in % response to CS during post-conditioning compared to pre-conditioning.

Before running ASR mice were exposed to 4 post-conditioning protocol sessions to extinguish FPS-learning.

Acoustic Startle Reflex (ASR)

ASR experiments were carried out between 08:30h and 17:00h in a modified version of the EMPReSS protocol for acoustic startle and pre-pulse inhibition (see www.eumorphia.org). Background noise (65 dB) was provided for ASR sessions, and bursts of white noise were used as startle pulses (stimulus duration: 40 msec). A session was initiated with 5 min acclimation period followed by five presentations of leader startle pulses (110 dB) that were excluded from statistical analysis.

Trial types included 7 different startle stimulus intensities (70, 80, 85, 90, 100, 110, 120 dB) and one NS trial, in which only the background noise was present to determine baseline movement of the animal. Each trial type was presented 10 times in random order, organised in 10 blocks, each occurring once per block. Inter-trail intervals (ITI) varied from 20-30 sec. Startle response was measured as the first peak-to-peak response with a minimum peak value of 50 arbitrary units occurring with a minimum wave onset latency of 20 msec and a minimum peak time of 30 msec following the onset of the startle stimulus.

Statistics

Data were expressed as mean + S.E.M. and statistically analysed using SPSS software (SPSS Inc, Chicago, USA) and the open source software R (http://www.r-project.org/). The accepted level of significance was $p < 0.05$. Normally distributed data were analysed by two-factorial analysis of variance (ANOVA), and for some mHB parameters where normality was violated, data were classified into 3 or 4 categories and loglinear models were fitted to explore the effects of strain, housing and their interaction. The Bonferroni test was used for post-hoc multiple comparisons with the t-test for normally distributed data or the χ^2 (chi-square)-test for data that was not normally distributed.

Significant intrastrain differences were only labelled with asterisks (*) in the figures in the case of a significant interaction between the factors. In case of a non-significant interaction, the 2-way ANOVA or the loglinear model were recalculated without interaction. The resulting p-values for the main factors were then taken as final result and mentioned in the text.

FPS data were first analysed by a 3-way ANOVA for repeated measures (independent factors *strain* and *housing*, dependent factor *conditioning*). In case of a non-significant interaction, the impact of housing conditions on learning was independently assessed for each strain by a 2-way ANOVA (factors *housing, conditioning*), which was also used for comparison of pre-conditioning performance of the two strains (factors *strain, housing*).

Acoustic startle response of each strain at 14 or 20 weeks of age was analysed by using the linear mixed-effects model (fixed factors: *housing*, *dB* and the interaction *housing x dB*; random factor: *animals*; dependent factor: *startle*). Startle threshold was also analysed by the linear mixed-effects model (fixed factor: *dB*; random factor: *animals*; dependent factor: *startle*). In case of a previous significant *housing x dB* interaction, the startle threshold was calculated separately for each housing condition. If this interaction was not significant, startle threshold was determined for each strain by pooling housing condition data. The threshold for response was defined as the stimulus level which consistently produced a significantly elevated response ($p < 0.05$) compared to the baseline response at 65 dB (background). To determine the impact of age on the startle response in each strain, pooled housing condition data of 14 and of 20 weeks were compared using the linear mixed-effects model (fixed factor: *age*, random factor: *animals*; dependent factor *startle*).

Results

Modified Hole Board

Locomotor activity (horizontal exploration): Concerning forward locomotion as measured by total distance travelled, housing conditions had a significant effect, which was the same in both strains [factor *housing*: $F(2,56) = 5.07$, $p < 0.01$; interaction *strain x housing*: $F(2,54) = 0.58$, n.s.]. Mice in conventional cages were more active than mice in IVCs **(Fig. 2a)**. Under all housing conditions, B6J mice were more active than C3H mice [factor *strain*: $F(1,56) = 165.19$, $p < 0.0001$].

Corresponding with total distance travelled, housing conditions had the same effect on mean velocity **(Fig. 2b)** in both strains [factor *housing*: $F(2,56) = 8.38$, $p < 0.001$; interaction *strain x housing*: $F(2,54) = 0.02$, n.s.]. Mice in conventional cages moved with higher mean velocity than mice in IVCs. Under all housing conditions, B6J mice moved with higher mean velocity than C3H mice [factor *strain*: $F(1,56) = 84.31$, $p < 0.0001$].

Anxiety-related behaviour: In both strains, housing conditions affected anxiety-related behaviour as measured by board entry parameters **(Figs. 2c-e)**. For the number of board entries **(Fig. 2c)**, the analysis revealed main effects of strain [factor *strain*: LR $\chi^2(3) = 79.36$, $p < 0.0001$] and housing [factor *housing*: LR $\chi^2(6) = 28.09$, $p < 0.001$], but no significant interaction between the factors [interaction *strain x housing*: LR $\chi^2(6) = 2e\text{-}10$, n.s.]. Eight out of 10 C3H mice in both types of IVCs did not make any board entry at all, compared with 2 out of 10 C3H mice in conventional cages. All B6J mice entered the board, but there was a stepwise reduction in the number of board entries from Conventional to IVC grid to IVC classic. Overall, B6J entered the board more often than C3H mice did.

Statistical analysis of the parameter latency to first board entry **(Fig. 2d)** revealed analogous strain and housing effects as the number of board entries. The impact of housing conditions on latency to first board entry was the same in both strains [factor *housing*: LR $\chi^2(4) = 15.07$, $p < 0.01$; interaction *strain x housing*: LR $\chi^2(4) = 0.44$, n.s.]: mice in IVCs entered the board later than mice in conventional cages, nevertheless this effect was significant for IVC classic in comparison to Conventional only. Overall, C3H mice started to explore the board later than B6J mice [factor *strain*: LR $\chi^2(2) = 37.82$, $p < 0.0001$].

Similarly to board entry frequency, time spent on board **(Fig. 2e)** was significantly affected by housing conditions in the same way in both strains [factor *housing*: LR $\chi^2(6) = 19.92$, $p < 0.01$; interaction *strain x housing*: LR $\chi^2(6) = 0.83$, n.s.]. Mice housed in IVCs spent less time

on board than mice housed in conventional cages. Overall, B6J mice spent more time on board than C3H mice [factor *strain*: LR $\chi^2(3) = 52.44$, $p < 0.0001$].

Exploratory activity: Concerning vertical (rearings) **(Fig. 2f)** and horizontal (hole explorations) **(Fig. 2g)** exploratory activity housing conditions had the same impact on both strains [rearings: factor *housing*: $F(2,56) = 9.27$, $p < 0.001$; interaction *strain x housing*: $F(2,54) = 0.95$, n.s.; holes explored: factor *housing*: LR $\chi^2(6) = 16.55$, $p < 0.05$; interaction *strain x housing*: LR $\chi^2(6) = 3e\text{-}11$, n.s.]. Mice in IVCs reared less and made fewer hole explorations than mice in conventional cages. Also these parameters reflect the higher activity level of B6J mice, which reared more and explored more holes than C3H mice [factor *strain*: rearings: $F(1,56) = 83.6$, $p < 0.0001$; holes explored: LR $\chi^2(3) = 79.36$, $p < 0.0001$].

Grooming: Housing conditions differentially affected the latency to grooming **(Fig. 2h)** in the two strains [interaction *strain x housing*: $F(2,54) = 4.11$, $p < 0.05$]. Latency to grooming was not influenced by housing conditions in C3H mice, in contrast to B6J mice. In both types of IVCs B6J mice showed lower latencies to grooming than in conventional cages. There were no general strain differences in the latency to grooming [factor *strain*: $F(1,54) = 0.62$, n.s.]. Neither significant main effects or *strain x housing* interactions were found for the time spent grooming and the frequency of grooming (data not shown) [time: factor *housing*: $F(2,56) = 0.47$, n.s.; factor *strain*: $F(1,56) = 3.4$, n.s.; interaction *strain x housing*: $F(2,54) = 0.35$, n.s.; frequency: factor *housing*: $F(2,56) = 0.54$, n.s.; factor *strain*: $F(1,56) = 2.85$, n.s.; interaction *strain x housing*: $F(2,54) = 0.7$, n.s.].

Fear Potentiated Startle

Analysis of pre-conditioning data showed that there was no strain difference in pre-conditioning baseline response to the CS, nor any influence of housing conditions on this parameter [factor *strain* $F(1,54) = 0.04$, n.s.; factor *housing* $F(2,54) = 0.97$, n.s.; interaction *strain* x *housing* $F(2,54) = 0.62$, n.s.]. There was also no significant interaction between strains, housing conditions and the FPS test paradigm [interaction *strain* x *housing* x *conditioning*: $F(2,54) = 1.2$, n.s.]. Analysis of main effects indicated overall strain and housing effects [factor s*train*: $F(1,54) = 6.45$, $p < 0.05$; factor *housing*: $F(2,54) = 4.2$, $p < 0.05$], and a clear overall learning effect [factor *conditioning*: $F(1,54) = 17.5$, $p < 0.001$]. There was no interaction between the two independent factors [interaction *strain* x *housing*: $F(2,54) = 1.6$, n.s.], but a tendential impact of housing conditions on learning [interaction

housing x *conditioning* F(2,54) = 3.1, p = 0.053]. Interestingly, as shown in Fig. 3, mice of both strains housed in IVC grid showed the highest post-conditioning increase in respect to pre-conditioning startle levels. There was a significant strain difference in FPS learning [interaction *strain* x *conditioning*: F(1,54) = 5.2, p < 0.05] **(Fig. 3)**.

FPS in C3H mice:

C3H mice did not learn with this FPS protocol [factor *conditioning*: F(1,27) = 3.04, n.s.] **(Fig. 3a)**, and there was neither a main effect of housing conditions [factor *housing*: F(2,27) = 1.76, n.s.], or an interaction [interaction *housing* x *conditioning*: F(2,27) = 0.73, n.s.].

FPS in B6J mice:

B6J mice did learn with this FPS protocol **(Fig. 3b)** [factor *conditioning*: F(1,27) = 14.9, p = 0.001], and statistical analysis also indicated a significant main effect of housing [factor *housing*: F(2,27) = 3.6, p < 0.05], but no interaction [interaction *housing* x *conditioning*: F(2,27) = 2.8, n.s.].

Acoustic Startle Reflex

ASR at 14 weeks of age: As expected, C3H mice showed a startle response curve [see Fig. 4a; factor *dB*: F(7,189) = 228, p < 0.0001]. Housing conditions influenced the acoustic startle response of C3H mice [factor *housing*: F(2,27) = 4.2, p < 0.05], although startle curve shape did not differ between housing conditions [interaction *housing x dB*: F(14,189) = 1.6, n.s.]. Comparisons revealed that IVCs increased the startle response in C3H mice as compared to C3H mice in conventional cages. There were no differences between IVC classic and IVC grid in this respect. Startle threshold analysis revealed a startle threshold of 80 dB in C3H mice at this age.

Also B6J mice showed a startle response curve at this age [see Fig. 4b; factor *dB*: F(7,189) = 207, p < 0.0001]. But housing conditions did not modulate the startle response of B6J mice [factor *housing*: F(2,27) = 1.1, n.s.], and startle curve shape was the same in all housing conditions [interaction *housing x dB*: F(14,189) = 0.9, n.s.]. The startle threshold of B6J mice was also 80 dB at this age.

ASR at 20 weeks of age: As before, C3H mice showed a startle response curve [factor *dB*: F(7,189) = 320, p < 0.0001], but at this age housing conditions differentially affected the curve shape [interaction *housing x dB*: F(14, 189) = 2.2, p < 0,01]. Separate analysis for each

housing condition revealed that mice in conventional cages had a startle threshold of 85 dB. In contrast, C3H mice in IVCs showed a lower startle threshold of 80 dB.
As at 14 weeks of age, also at this age B6J mice showed a startle response curve [factor *dB*: $F(7,175) = 84.5$, $p < 0.0001$] and startle response was independent of housing conditions [factor *housing:* $F(2,25) = 0,7$, n.s.; interaction *housing x dB*: $F(14,175) = 0.5$, n.s.]. Startle threshold analysis also revealed a startle threshold of 80 dB in B6J mice at this age.

Age effect on the startle response: Age had no influence on the magnitude of startle responses in C3H mice [factor *age*: $F(1,449) = 0.003$, n.s], i.e. response magnitudes did not differ between 14 and 20 weeks of age. In contrast, startle response magnitudes of B6J mice were modulated by age [factor *age*: $F(1,446) = 48.3$, $p < 0.0001$]. At the age of 20 weeks, B6J mice startled less than at the age of 14 weeks.

Health status

At the end of the experiment all mice from both the C3H and B6J strains were seronegative to MHV and MMV independent of the caging system used. With respect to MPV, the number of seropositive C3H mice totalled 4, 1 and 1 in Conventional, IVC classic and IVC grid cages, respectively. All mice from the B6J strain showed no antibodies to MPV.

Discussion

The potential impact of housing conditions on mouse physiology and behaviour is of increasing interest, particularly if the goal is to attribute phenotypic differences to experimentally induced alterations in the mouse genome on a large scale. To this end, the comprehensive analysis of mutant mouse lines will likely need to be shared between different facilities, creating a need for information about comparability of phenotypic data.

It is known that environmental factors as well as genetic background influence behavioural phenotypes (for review see Crawley et al. 1997; Wolfer and Lipp 2000). It was not known whether an IVC housing environment can affect behavioural phenotype results, and if it does, to what extent. To address this issue, in the present study we analysed emotionality and fear learning in male C3HeB/FeJ and C57BL/6J mice singly housed in IVCs in comparison to mice housed in the same room in conventional type II cages of the same size.

Spontaneous Behaviour

IVC housing reduced locomotor (total distance travelled, mean velocity) and exploratory activity (rearing, hole exploration), and increased anxiety-related behaviour (decline in entries on board, increase in latency to first entry and reduction of time on board) in both strains. IVC housing reduced the latency to grooming behaviour in B6J mice, but not in C3H mice. Grooming behaviour is a response often provoked by novelty (Dunn et al. 1981), which has an adaptive de-arousal function following stress (Spruijt et al. 1992). Thus, one may regard an earlier display of grooming behaviour as an indicator of a lower arousal level. However, this parameter must be treated with care, firstly, because it was shown that grooming behaviour differs in inbred strains (Kalueff and Tuohimaa 2005), consistent with our findings. Secondly, because grooming behaviour has also been related to emotionality (Hoover-Plow et al. 2001), and the earlier display of grooming by IVC housed B6J mice might also be regarded as reflecting increased anxiety.

The “grid” did not alter the impact of IVC housing on spontaneous behaviour in C3H mice, and only very mildly modulated it in B6J mice in activity- and anxiety-related parameters. Taken together, these data show that firstly, the IVC system has an activity-reducing and anxiety-increasing effect on singly housed male C3H and B6J mice. Secondly, the IVC system reduces grooming latency in singly housed male B6J mice, and thirdly, the “grid” is not sufficient to counteract these effects of IVC housing.

The last point suggests that the attenuation of external environmental stimuli in IVCs has a stronger effect on unconditioned behaviors of singly housed male C3H and B6J mice than the reduction of climbing facilities. A possible explanation for these findings is that mice in IVCs are so little exposed to environmental stimuli that for these mice the transition to the test environment makes a bigger difference than for mice housed in conventional cages. To our knowledge, up to now there is no systematic investigation concerning the impact of housing in acoustically attenuated/deprived conditions on murine behaviour, although Strasser and Dixon (1986) have shown that acoustic deprivation of normal hearing mice diminished active tracking in the resident/intruder paradigm.

Independent of housing conditions, B6J mice were more active than C3H mice, which was reflected by increased total distance travelled, mean velocity, and frequencies of board entry, rearing and hole exploration. Additionally, B6J mice showed less anxiety-related behaviour than C3H mice since they spent more time in the exposed area of the mHB, namely the board. These results are consistent with previous reports on behaviour of these two strains. Data from tests used for evaluation of anxiety suggest that C57BL/6 mice are less anxious than C3H mice (Bouwknecht and Paylor 2002; Ducottet and Belzung 2005). Concerning the C3HeB/FeJ substrain of C3H mice, this has so far been restricted to locomotor and anxiety-related behaviour (Tarantino et al. 2000). The C3HeB/FeJ substrain carries the retinal degeneration gene Prd6b and mice of both sexes progressively loose retinal function with an onset of one to two months of age (Chang et al. 2002). This may account for the reduced exploratory and increased anxiety-related behaviour of C3H in comparison to B6J mice.

In contrast to C3H, B6J mice are known for high spontaneous locomotor activity and high baseline exploratory tendency in response to nonsocial novelty (Crawley and Davis 1982; Nikulina et al. 1991). B6J mice also showed high emotional reactivity in the elevated plus-maze with an open arms avoidance index of >90% (Trullas and Skolnick 1993), which is in line with our present findings of the level of avoidance of the board, the exposed area in the middle of the test arena. In our study, the level of time on board in B6J mice differed between 8% (IVC classic) and 13% (Conventional).

Sensorimotor Function

IVC housing increased startle response magnitudes in C3H mice at 14 weeks, and reduced startle threshold at 20 weeks of age. An obvious explanation is that IVC housed mice react more sensitively to acoustic stimuli as they have been raised in an acoustically attenuated environment as compared to Conventional. The enhancement of the acoustic startle response

in IVC housed C3H mice is likely related to the described anxiety-increasing effect of IVC housing measured in the mHB. This assumption is in line with reports about a correlation of anxiety and startle response levels in mice and humans (Prehn et al. 2005; Shum et al. 2005). In contrast, IVC housing did not affect sensorimotor function of B6J mice at both ages tested. Nevertheless, in comparison to C3H mice, the impact of IVC housing goes in the same direction in B6J mice at 14 weeks of age (see Fig. 4b), although statistically not significant. This may be due to the fact that, firstly, B6J are less anxious than C3H mice, secondly, B6 mice suffer from genetically determined progressive sensorineural hearing loss (Johnson et al. 1997), which might already affect them at this age, and thirdly, blind can hear better than normal-seeing individuals (Niemeyer and Starlinger 1981). Altogether, this leads to the hypothesis that the anxiety-increasing effect of IVC housing facilitates a *significant* increase in startle response in blind C3H mice as compared to B6J mice.

In both strains acoustic startle reflex assessment revealed a startle threshold of 80 dB at 14 weeks of age. Age reduced startle response magnitude in B6J, but not in C3H mice. Notably, this is not reflected by an increase of startle threshold, but a flattening of the startle response curve. Thus, because of their sensorineural hearing loss, tests relying on auditory function are best performed until the age of 3 - 4 months in B6J mice, maybe even earlier.

Fear Learning

In contrast to B6J, the FPS protocol used in this study was not sufficient to induce fear learning in C3H mice, independent of housing conditions. Inbred strains have been shown to differ in the rate of acquisition of conditioned fear, depending on the number of fear-conditioning trials needed, and can also differ in shock intensities needed for successful conditioning (Falls 2002). To our knowledge cognitive function of this particular C3H substrain (C3HeB/FeJ) has not been investigated so far. However, such information is needed because this substrain was used for a large-scale ENU mutagenesis screen, in which several behavioural mutants have been found which are awaiting their detailed CNS function analysis (Hrabe de Angelis et al. 2000). Here we showed that compared to B6J mice, C3H mice are impaired in acquisition of Fear Potentiated Startle. More work is needed to establish an FPS protocol for C3HeB/FeJ mice, which is beyond the scope of this study.

Interestingly, in B6J mice fear potentiation of the startle response was most pronounced for mice housed in IVC grid and less pronounced in IVC classic (see Fig. 3b). Notably, fear potentiation in IVC grid was superior to that in Conventional. These results indicate that the

FPS protocol is not suitable for studies with singly housed male B6J mice housed in IVC classic. Thus, either improved housing conditions, e.g. by the use of “grids”, or an improved FPS protocol are warranted in this case. Demands for improved housing conditions are supported by the finding that cued fear conditioning was enhanced in mice with enriched experience (Rampon et al. 2000). Although our IVC system did not allow for the measurement of activity levels in the home cage, our results of improved FPS performance for singly housed B6J with climbing facilities in the home cage are in agreement with previous reports that increased exercise facilitates learning (Vaynman and Gomez-Pinilla 2005). Furthermore, our data indicate a positive effect of housing in acoustically attenuated conditions concerning external stimuli in combination with climbing facilities on FPS performance in singly housed male B6J mice. This interpretation is based on the fact that fear potentiation of B6J males in IVC grid was superior to that in Conventional.

Additional considerations

For data interpretation, it should be taken into account that mice in this study were tested repeatedly, therefore, carry-over effects are likely to occur (McIlwain et al. 2001). Naïve animals per each test would have eliminated a possible bias resulting from the test order. On the other hand, for high-throughput phenotyping - regarding time as well as cost efficiency - a batch of a mouse line has to run through a test-battery. To bias the animals as little as possible, the test order is therefore from the least to the most aversive/stressful one. Further, a test-battery comprises two additional advantages: it provides intra-individual comparability across the tests (intra- and interdisciplinary) and lowers the number of animals used, which in turn is welcome regarding animal welfare.

Although there is an increasing interest in the question whether different environments affect group variability (Tsai et al. 2002; Wolfer et al. 2004), the (mean) absolute values of the parameters measured are equally important for behavioural analysis of mutant mouse lines. The rationale for this is that first, monitoring absolute values of control animals allows the detection of shifts in baselines and second, for behavioural phenotyping an adequately high baseline performance is indispensable. Otherwise, phenotyping on the basis of extreme, minimal or maximal, baseline levels is likely to complicate the detection of genetic effects. Applied to our study, the conclusion drawn is that alterations in anxiety-related parameters are the most difficult ones to be identified on B6J and C3H background when male mice are singly housed in IVCs. This is based on the fact that anxiety-related parameters exhibited low mean values in both strains when IVC housed, especially in C3H mice. In contrast, although

IVC housing also reduced locomotor activity, the remaining activity level would still be high enough to detect alterations in both directions.

Conclusions

The present findings show that IVC housing can affect behavioural performance. Single housing of male mice in the IVCs used in this study renders the detection of genetic effects which increase basal anxiety-related behaviour impossible on a C3H genetic background, and aggravates it on a B6J genetic background. The use of "grids" in these IVCs does not counteract this effect in C3H mice, and hardly improves it in B6J mice, suggesting that climbing facilities are of minor importance for the impact of IVC housing on the unconditioned behaviours measured in this study. We conclude that this impact is mainly due to the IVC feature of acoustical attenuation/deprivation from external stimuli. However, for testing fear learning as measured by FPS, IVC grid proved superior to Conventional, indicating advantages of IVC housing in combination with exercise possibilities for FPS performance. It is unclear whether this effect extends to other cognitive functions relying on other sensory modalities. Our finding of a possible impact of variations in IVC cage structure on mouse behaviour corroborates recent findings demonstrating that different IVC cage features (cardboard roll or mouse house) can differentially affect mouse behavioural parameters (Tucci et al. 2006).

Whether these findings also hold true for female mice and for group-housed mice needs to be investigated. Likewise, since different IVC systems vary in cage size, structure and functionality, the kind of IVC system may also make a difference. Since the ultimate goal of mouse functional genomics is to identify genetically modified mice as model systems for the investigation of molecular mechanisms of human diseases, knowledge about the impact of different housing and handling conditions as well as test protocols on phenotyping results is needed for their appropriate choice.

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Figure legend:

Fig. 1: Photographs of the lower surface of the three types of lids of the type II cages used for conventional housing (a) and for housing in individually ventilated cages (IVCs, b, c) in the present experiment as seen from the mouse's point of view. The food hoppers are shown in the upper half of the lids. a: „Conventional", a standard wire bar lid commonly used for plastic shoe box-type II mouse cages. b, „IVC grid", standard solid Biozone™ IVC lid as shown in c, but with a custom-made rectangular wire bar ("grid") welded to the lower part of the lid covering the smooth surface. c, „IVC classic", standard solid Biozone™ IVC lid as provided by the manufacturer with smooth, untextured lower surface. The perforations in the rounded rectangular fields at the upper and lower areas of the lids shown in b and c facilitate ventilation of the IVCs through the solid lids.

Fig. 2: Impact of housing conditions on unconditioned behaviour in C3HeB/FeJ and C57BL/6J male mice in the *modified Hole Board* paradigm at the age of 9 weeks; locomotor activity as measured by total distance travelled (a) and mean velocity (b); anxiety-related behaviour indicated by board entries (c), latency to first board entry (d) and time on board (e); exploratory activity as measured by rearings (f) and hole exploration (g) and (h) arousal behaviour. Data are expressed as means + S.E.M., n = 10. Intrastrain specific differences are labelled with asterisks in the case of significant differences. ** $p < 0.01$ IVC grid or IVC classic vs. Conventional (latency to grooming). In case of a non-significant interaction between strain and housing (total distance travelled, mean velocity, board entries, latency to first board entry, time on board, rearings and hole exploration) the p-values are mentioned in the text.

Fig. 3: Impact of housing conditions on learning abilities in the *Fear Potentiated Startle* paradigm in C3HeB/FeJ (a) and C57BL/6J (b) male mice at the age of 10 weeks. Data are expressed as means + S.E.M., n = 10.

Fig. 4: Impact of housing conditions on *Acoustic Startle Reflex* in C3HeB/FeJ (a,c) and C57BL/6J (b,d) male mice at the age of 14 (a,b) and 20 weeks (c,d). Data are weight-normalized and expressed as means + S.E.M., n = 9-10. °° $p < 0.01$ threshold Conventional, ~ $p < 0.05$ threshold IVC grid, ++ $p < 0.01$ threshold IVC classic.

Fig. 1

a

b

c

Fig. 2

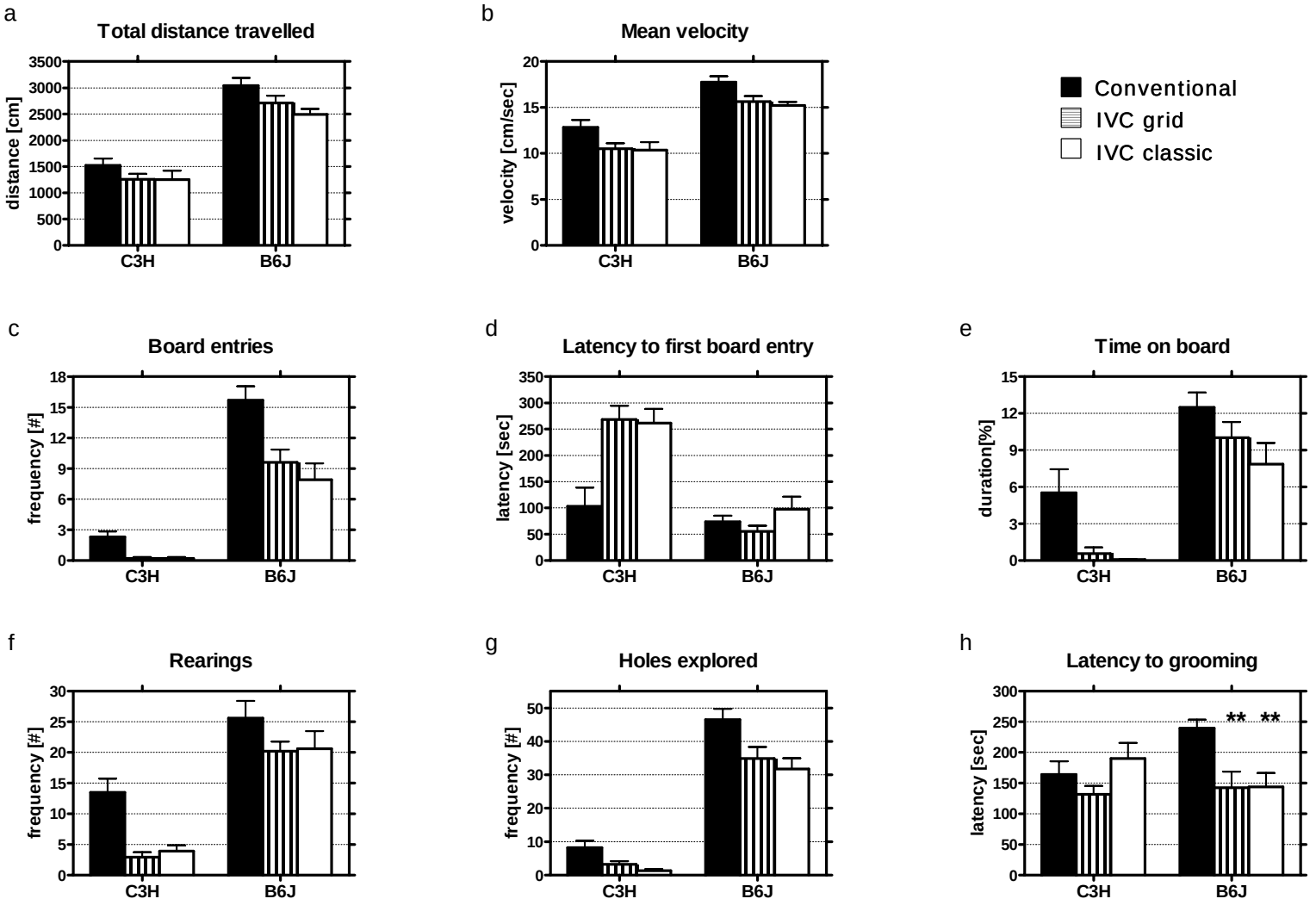

a
Total distance travelled
distance [cm]
3500
3000
2500
2000
1500
1000
500
0
C3H
B6J
b
Mean velocity
velocity [cm/sec]
20
15
10
5
0
C3H
B6J
Conventional
IVC grid
IVC classic
c
Board entries
frequency [#]
18
15
12
9
6
3
0
C3H
B6J
d
Latency to first board entry
latency [sec]
350
300
250
200
150
100
50
0
C3H
B6J
e
Time on board
duration[%]
15
12
9
6
3
0
C3H
B6J
f
Rearings
frequency [#]
30
25
20
15
10
5
0
C3H
B6J
g
Holes explored
frequency [#]
50
40
30
20
10
0
C3H
B6J
h
Latency to grooming
latency [sec]
300
250
200
150
100
50
0
** **
C3H
B6J

Fig. 3

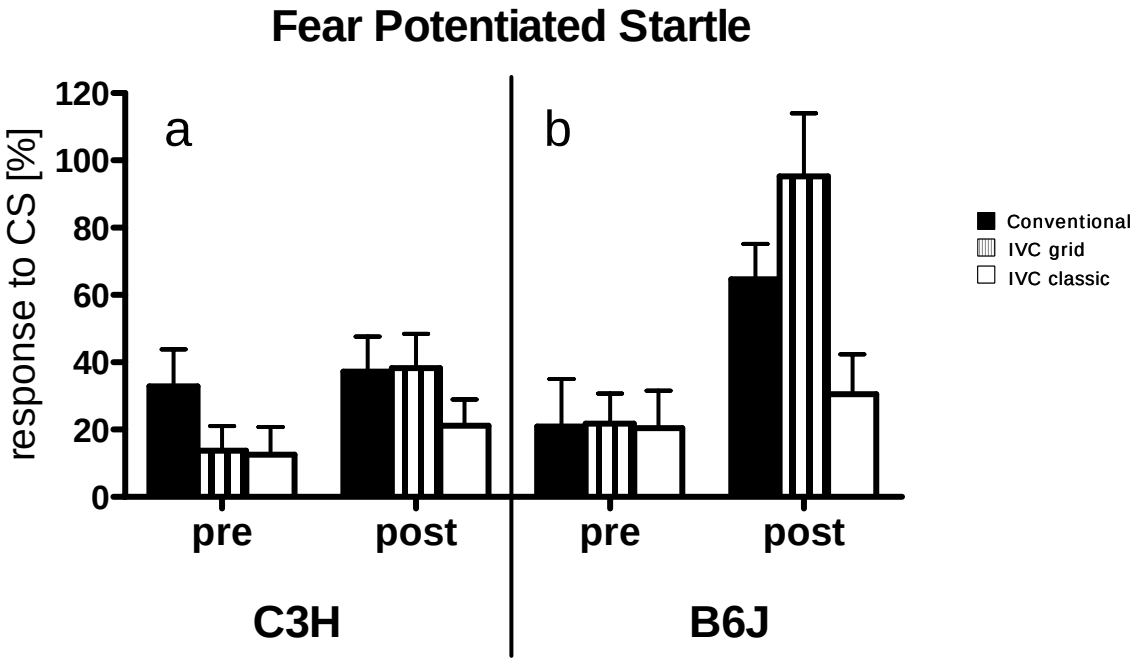
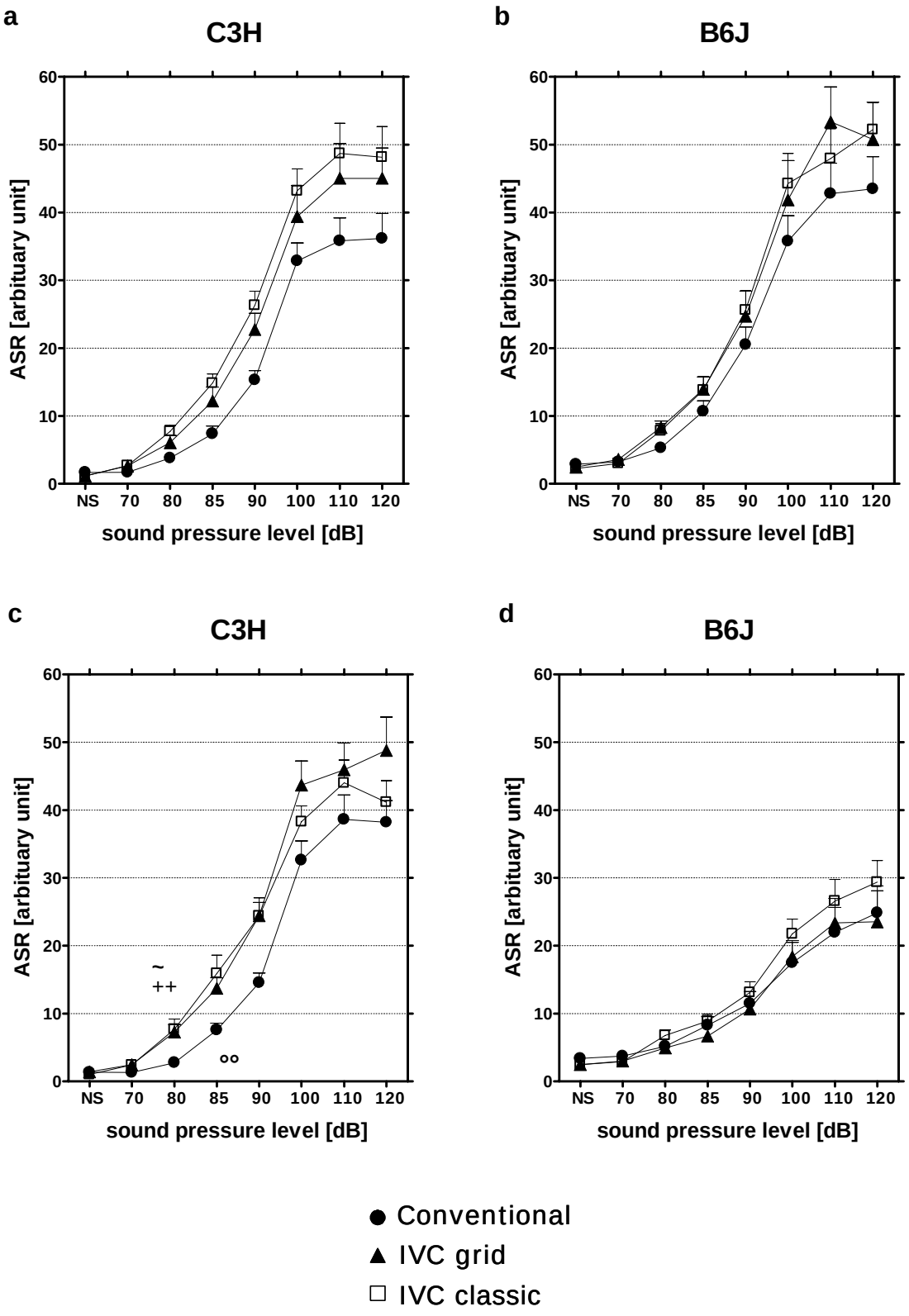

Fear Potentiated Startle
response to CS [%]
120
100
80
60
40
20
0
a
b
pre
post
pre
post
C3H
B6J
Conventional
IVC grid
IVC classic

Fig. 4


a
C3H
b
B6J
c
C3H
d
B6J
ASR [arbituary unit]
sound pressure level [dB]
NS 70 80 85 90 100 110 120
0 10 20 30 40 50 60
~
++
oo
Conventional
IVC grid
IVC classic