Chapter 4

STRESSOR SUSCEPTIBILITY IN LOW AND HIGH AGGRESSIVE MICE: EFFECTS OF TIME AND OF TYPE OF OPPONENT IN SENSORY CONTACT MODEL

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Abstract

It was previously shown that male wild house-mice selectively bred for long attack latency (LAL) and short attack latency (SAL) showed a differential response to continuous sensory (except for tactile) contact with an aggressive (SAL) male for a period of 25 days. After this prolonged exposure to a SAL male, only the LAL mice displayed stress symptoms, such as decreased body weight, increased corticosterone levels, and lower hippocampal mineralocorticoid receptor (MR) mRNA expression. In the present study, the hypothesis was tested that (i) the differential stress symptoms of LAL and SAL mice are already present after 5 days of sensory contact stress, and that (ii) the magnitude of the response to sensory contact is determined by the type of opponent (i.e. a high-aggressive SAL male vs. a low-aggressive LAL male vs. isolation). The results show that after 5 days of continuous exposure to a SAL male, LAL mice had high levels of corticosterone, adrenal hypertrophy, and increased mRNA expression of hypothalamic CRH and hippocampal dentate gyrus glucocorticoid receptor (GR), while hippocampal CA1 serotonin-1A (5-HT$_{1A}$) receptor was decreased. In contrast to LAL mice, markers of the HPA system in SAL mice suggest that 5 days of SAL exposure had only transient effects. With respect to the type of opponent, it was shown that isolation of in particular the LAL mice, caused profound changes in several peripheral parameters. However, when exposed to a SAL male, LAL mice showed higher body weight loss, higher corticosterone levels (at day 2), adrenal hypertrophy and lower seminal vesicle mass than LAL mice of any other group. In SAL mice, living opposite a LAL male produced higher ACTH levels and adrenal hypertrophy than all other SAL groups. In conclusion, 5 days of continuous exposure to the sensory contact stressor caused differential stress symptoms in LAL and SAL mice that depended beyond isolation on the type of opponent. LAL mice exposed to a SAL male showed the most severe stress symptoms, which at a central level were however distinct from the symptoms induced after 25 days of exposure to the sensory contact stressor.
Introduction

To study the role of individual differences in susceptibility for stress-related psychopathology, stress responsiveness was investigated in two mouse lines showing distinctly different coping styles. These wild house-mice are selectively bred for long attack latency (LAL) and short attack latency (SAL). Several studies have shown that this genetic property can be extended to a passive coping style (LAL) and an active coping style (SAL) towards environmental stimuli (Benus et al., 1989, 1991a,b; Sluyter et al., 1996).

Recently, coping style was found to be associated with differences in the serotonergic (5-HT) system (Korte et al., 1996; Van Riel et al., 2002) and differences in the regulation of the Hypothalamic-Pituitary-Adrenal (HPA) system under basal and acute stress conditions (Veenema et al., 2003a). LAL mice had a lower hippocampal 5-HT$_{1A}$ receptor gene expression and function than SAL mice (Korte et al., 1996; Van Riel et al., 2002). Furthermore, the corticosterone output in LAL mice was found to be more sensitive to ACTH, but showed less day-night variation than in SAL mice. In addition, LAL mice showed a higher and prolonged stress-induced increase in plasma corticosterone compared to SAL mice (Veenema et al., 2003a). These results suggest a line difference in stressor susceptibility.

Indeed, we could recently demonstrate that genetic selection for coping style predicts stressor susceptibility by using a sensory contact model (Veenema et al., 2003b). In this model, male mice were living in sensory (visual, auditory, and olfactory but not tactile) contact with an aggressive (SAL) male for 25 days. At this time point, stress symptoms were found only in LAL males, as indicated by decreased body weight, elevated plasma ACTH and corticosterone levels and lower hippocampal mineralocorticoid receptor (MR) mRNA expression (Veenema et al., 2003b).

In view of this large line difference observed at day 25, it is of interest to study the development of this difference in the course of time. In addition, it is relevant to know to what extent the type of opponent determines the magnitude of the effects. It was hypothesized, that (i) the differential response between LAL and SAL mice is already present after 5 days of sensory contact with a SAL male, and (ii) the magnitude of the response to sensory contact is determined by the type of opponent (i.e. the aggressive SAL male). The effect of sensory contact with a SAL male for 5 days was determined for body weight, plasma corticosterone and ACTH, organ weights and the mRNA expression of MR, glucocorticoid receptor (GR), and 5-HT$_{1A}$ receptor in the hippocampus, and GR and corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) of the hypothalamus (Experiment 1). To investigate the effect of type of opponent, LAL and SAL males were either housed single (isolation), in sensory contact with a LAL male or in sensory contact with a SAL male (Experiment 2). In experiment 2, body weight, plasma corticosterone and ACTH and several organ weights were measured as well as the behaviour of the mice that were housed in the partition cage.
Methods

Animals

The two mouse lines, which were genetically selected for attack latency, originated from a colony of wild house-mice (*Mus musculus domesticus*) maintained at the University of Groningen, The Netherlands, since 1971. The mice were housed in perspex cages (17 x 11 x 13 cm) in a room with a 12:12 light/dark cycle (lights on from 0030 to 1230). Standard laboratory chow and water was available *ad libitum*. The mice were weaned at 3-4 weeks of age, and were paired male-female at the age of 6-8 weeks. At the age of 92-100 days, male mice were tested for their attack latency as described by Van Oortmerssen and Bakker (1981). Briefly, genetically selected LAL and SAL males are confronted with a standard non-aggressive opponent male of an inbred albino strain (MAS-Gro) at the border of their home cage. The time it takes before a SAL or LAL mouse attacks the non-aggressive opponent is measured on three consecutive days. The attack latency score is the mean of these daily scores. Neither LAL nor SAL mice experienced a social defeat. Only non-attacking LAL mice and SAL mice with an attack latency of less than 50 s were used for the experiments. The SAL males came from the 63-65th generation of selection, the LAL males from the 38-40th generation and were at the age of 17 weeks (± 2 weeks). All experiments were in accordance with the regulations of the Committee for Use of Experimental Animals of the University of Groningen (DEC nr. 2326).

Experiment procedures

In experiment 1 (see Fig. 1), it was investigated whether living in sensory contact with an aggressive (SAL) male for 5 days induced a differential response between LAL and SAL mice. In this paradigm a LAL (n = 8) or SAL (n = 7) male was living for 5 days in a partition cage (75 x 29 x 27 cm) opposite an aggressive (SAL) male. A perforated (diameter of 5 mm) transparent partition separated the cage into two equal halves and allowed the mice to see, hear and smell each other, but prevented physical contact. Control LAL and SAL males (n = 8 per line) were housed under standard conditions with a female in perspex cages (17 x 11 x 13 cm).

In experiment 2 (see Fig. 1), it was studied whether the magnitude of the response to sensory contact is determined by the type of opponent. To study this, a LAL or SAL male was living for 5 days in a partition cage (75 x 29 x 27 cm) either single (single: LAL n = 8; SAL n = 9), opposite a LAL male (LAL opp: n = 6 per line), or opposite a SAL male (SAL opp). The SAL opp groups and the control groups in this experiment were the same groups as used in experiment 1.

For both experiments, body weight was measured at all experimental days (day 1 till day 5). At day 2, between 0800 and 0900 (4½ - 3½ h before the dark phase), blood was obtained from the mice by a tail cut to measure plasma corticosterone concentrations. In experiment 2, behaviour of the mice housed in the partition cage was determined on day 1 till day 4. At day 5, all mice were decapitated under CO₂ anaesthesia between 0800 and 0900, trunk blood was collected for corticosterone and ACTH measurements and several organs were removed and weighed. For experiment 1, brains were rapidly removed, quickly frozen in p-heptane (-60°C) and stored in –80°C for subsequent *in situ* hybridisation.
Housing conditions: LAL male  

Experiment 1
Control
SAL opp

Experiment 2
Control
Single
LAL opp
SAL opp

Fig. 1. Schematic illustration of the housing conditions in experiment 1 and 2. In experiment 1, the effect of 5 days of living opposite a SAL male in the sensory contact model was examined. In experiment 2, the effect of type of opponent (i.e., SAL male, LAL male, single) in the sensory contact model was examined. Control mice were housed under standard conditions with a female. In the figure, the experimental LAL or SAL males are shown in the right halve of the partition cage, and the opponent males in the LAL opp and SAL opp treatment groups, are shown in the left halve of the cage.
Radioimmunoassay for corticosterone and ACTH

Blood was collected in chilled tubes containing EDTA for determination of corticosterone and ACTH levels. Blood samples were centrifuged at 2600 g for 10 min at 4°C. Plasma samples were stored at –20°C until assayed. Plasma corticosterone was determined in duplo using a commercially available radioimmunoassay (Mouse Corticosterone RIA Kit, ICN Biomedicals, Costa Mesa, CA, USA). The detection limit of the assay was 3 ng corticosterone/ml with an intra-assay variance of 4.4% and inter-assay variance 6.5%. A double-antibody radioimmunoassay (ACTH RIA kit, Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) with intra-assay and inter-assay variances of 3.2% and 7.8% was used to measure plasma ACTH. The detection limit of the assay was 1 pg ACTH/ml.

In situ hybridisation

To determine the effect of sensory contact stress with a SAL male in experiment 1, in situ hybridisation was performed on brain slices of control LAL and SAL males (n = 8 per line) and of LAL and SAL males living opposite a SAL male (n = 5 per line). Brain tissue sections of 14 µm were cut on a cryostat and thaw-mounted on poly-L-lysine coated slides and stored at –80°C until the time of hybridisation. For MR and GR, riboprobes were generated from linearized constructs containing the respective cDNA’s in pBluescript. A 500bp SalI-HindIII fragment of exon 2 of the mouse gene was used for GR and a 1.2 kb Ncol-EcoRI fragment of the mouse MR exon 2 for the MR (courtesy of Dr. T. Cole). The cRNA from CRH was transcribed from a 1-kb cDNA insert in pGEM 4 containing full-length coding region of rat CRH. Riboprobe in situ hybridisations (MR, GR, CRH) were performed as described in Veenema et al. (2003b). For the 5-HT₁A receptor probe we used the following specific oligonucleotide: 5’-TGG-AGA-TGA-GAA-AGC-CAA-TGA-GCC-AAG-TGA-GCG-AGA-TCA-GCG-CAG-3’. To control for specificity of hybridisation, an oligonucleotide was used that was identical except for 6 point mutations, evenly spaced at approximately every 7 nucleotides; 5’-TGT-AGA-TGA-TAA-AGC-AAA-TGA-TCC-AAG-GGA-GCG-CGA-TCA-TCG-CAG-3’. Oligonucleotide in situ hybridisation (5-HT₁A receptor) was performed as described in Meijer et al. (2000). Hybridised slices were exposed to a X-Omat AR film (Kodak, Rochester, NY, USA) for 1 day (MR), 4 days (GR in PVN), 6 days (CRH), 9 days (hippocampal GR) or 13 days (5-HT₁A receptor). Optical density was determined by using a digital image analysis system (analySIS, soft imaging system). For hippocampal MR, GR and 5-HT₁A receptor mRNA expression, the optical density of CA1, CA2 (only MR), CA3 and dentate gyrus was determined in three hippocampal sections of each mouse. The values of these sections were averaged for each mouse. The optical density of a small area between the CA1 and dentate gyrus was used for tissue background. For GR and CRH mRNA expression in the PVN the optical density was determined in three sections of each mouse. The values of the three sections were averaged for each mouse. A nonhybridized region outside the PVN was measured for tissue background.

Behaviour in the partition cage

In experiment 2, behaviour in the partition cage was determined in LAL and SAL mice housed single (n = 6 per line), opposite a LAL male (n = 6 per line) or opposite a SAL male (n = 8 for LAL and n = 7 for SAL), using a modified scanning procedure (Keeney and Hogg, 1999). Their position in the cage relative to the partition was recorded as well as
their behaviour (inactivity, exploration, grooming, eating/drinking and social interaction with the male opponent). Each mouse was scanned every 5 minutes for one hour, resulting in a maximum score of 13 per session. This procedure was carried out between 1100 and 1200 (late light phase) at day 1, day 2, and day 3 and between 1530 and 1630 (early dark phase) at day 1, day 2, and day 4.

 Statistical analysis

Relative body weight and plasma corticosterone concentrations were analysed using analysis of variance (ANOVA) for repeated measures. Univariate ANOVA was used to determine treatment, line, and interaction effects for plasma ACTH concentrations and relative organ weights. In experiment 1, the mRNA expression of MR, GR, CRH and 5-HT\textsubscript{1A} receptor in several brain regions was analysed with an univariate ANOVA. In experiment 2, behaviour was analysed using ANOVA for repeated measurements. When significance was revealed, appropriate pairwise comparisons (LSD test) were done based on the estimated marginal means. For all tests, the software package SPSS (version 11) was used, and the level of significance was \( P < 0.05 \). Data are presented as mean ± S.E.M.

 Results

 Experiment 1: Effect of sensory contact stress with a SAL male for 5 days

Body weight (Fig. 2)

Body weight was measured daily, starting just before mice were placed in the partition cage (day 1) till day 5. Body weight at day 1 was set at 100%. Repeated measures ANOVA revealed a main effect for treatment \( (F_{1,26} = 53.729, P < 0.001) \) and a time × treatment interaction \( (F_{1,26} = 18.841, P < 0.001) \). LAL and SAL mice housed in the partition cage showed a significantly decrease in relative body weight from day 2 till day 5 compared to control LAL and SAL mice, respectively \( (P \text{ at least < 0.005, Fig. 2A,B).} \)

Corticosterone (Fig. 3A,B)

Plasma corticosterone was measured by obtaining blood (by a tail cut at day 2 and by decapitation at day 5) between 0800 and 0900 (4½-3½ h before the dark phase). A main effect for treatment \( (F_{1,26} = 6.521, P < 0.05) \) and a treatment × line interaction \( (F_{1,26} = 31.032, P < 0.001) \) was found. LAL mice housed opposite a SAL male showed significantly higher corticosterone concentrations at day 2 \( (P < 0.01 \text{ vs. SAL mice, } P < 0.001 \text{ vs. LAL control, Fig. 3A}) \) and day 5 \( (P < 0.005 \text{ vs. SAL mice, } P < 0.001 \text{ vs. LAL control, Fig. 3B}) \). Control LAL mice had lower corticosterone concentrations than control SAL mice at day 2 \( (P < 0.01, \text{Fig. 3A}). \)

ACTH (Fig. 3C)

Plasma ACTH concentrations were measured at day 5 by obtaining blood by decapitation between 0800 and 0900 (4½-3½ h before the dark phase). Univariate
ANOVA showed a significant line effect ($F_{(1,26)} = 6.855$, $P < 0.05$). LAL mice showed lower ACTH concentrations than SAL mice resulting in a significant difference in mice housed opposite a SAL male ($P < 0.05$, Fig. 3C).

**Fig. 2.** Effect of sensory contact with a SAL male for 5 days on the relative body weight of LAL (A) and SAL (B) mice. At day 1, mice were housed in the partition cage opposite a SAL male (SAL opp) or were put in a novel cage (control). Body weight at day 1 was set at 100% for each mouse. * $P$ at least < 0.005 vs. control, pairwise comparisons (LSD test) following repeated measures ANOVA.

**Fig. 3.** Effect of sensory contact with a SAL male on plasma corticosterone concentrations at day 2 (A) and day 5 (B) and ACTH concentrations at day 5 (C) in LAL and SAL mice. * $P < 0.001$ vs. control, # $P$ at least < 0.05 vs. SAL, pairwise comparisons (LSD test) following univariate ANOVA.
Organ weights (Fig. 4)

After the experimental period of 5 days, all mice were decapitated and several organs were weighed as possible peripheral indicators of altered HPA axis functioning. All organ weights were corrected for body weight at day 5.

Thymus weight (Fig. 4A).

A significant treatment ($F_{(1,27)} = 29.626, P < 0.001$) and line ($F_{(1,27)} = 10.473, P < 0.005$) effect was found. LAL and SAL mice living opposite a SAL male showed significantly lower thymus weights than control LAL and SAL mice ($P \leq 0.001$). Both LAL groups had higher thymus weights than the SAL groups ($P < 0.05$).

Adrenal weight (Fig. 4B).

A significant treatment effect was found ($F_{(1,27)} = 6.415, P < 0.05$). Only LAL mice housed opposite a SAL male had significantly higher adrenal weights than control LAL mice ($P < 0.05$). No line effect was found.

Seminal vesicle weight (Fig. 4C).

A significant treatment ($F_{(1,26)} = 16.827, P < 0.001$) and line ($F_{(1,27)} = 8.950, P < 0.01$) effect was found. LAL mice housed opposite a SAL male had significantly lower seminal vesicle weights than control LAL mice ($P < 0.005$) and than SAL mice housed opposite a SAL male ($P < 0.05$).

Spleen and testes weights.

No treatment or line effects were found (data not shown).

![Fig. 4. Effect of sensory contact with a SAL male for 5 days on relative organ weights [organ weight (mg)/body weight (g)] of LAL and SAL mice after the experimental period of 5 days. (A) Relative thymus weight. (B) Relative adrenal weight. (C) Relative seminal vesicle weight. * $P$ at least $< 0.05$ vs. control, # $P < 0.05$ vs. SAL, pairwise comparisons (LSD test) following univariate ANOVA.](image-url)
In situ hybridisation (Table 1 and 2)

In situ hybridisation was performed to determine the effect of 5 days of sensory contact with a SAL male on the expression levels of CRH mRNA, GR mRNA, MR mRNA and 5-HT\textsubscript{1A} receptor mRNA.

**CRH mRNA expression (Table 1).**

A treatment effect was found ($F_{(1,20)}=12.338$, $P < 0.005$) for CRH mRNA expression in the PVN. Only LAL mice housed opposite a SAL male showed a significantly higher expression of CRH mRNA ($P < 0.01$ vs. LAL control). No line effect was found.

**GR mRNA expression (Table 1).**

A treatment effect was found for GR mRNA in the dentate gyrus of the hippocampus ($F_{(1,21)}=8.675$, $P < 0.01$). LAL mice living opposite a SAL male showed a higher expression of GR mRNA in the dentate gyrus ($P < 0.01$ vs. LAL control). No treatment or line effect was observed for GR mRNA in the PVN nor in hippocampal CA1 and CA3 regions (CA3 data not shown).

**MR mRNA expression (Table 1).**

No significant line or treatment effect was found for the expression of MR mRNA in any subregion of the hippocampus (data of CA2 and CA3 regions not shown).

**5-HT\textsubscript{1A} receptor mRNA expression (Table 2).**

A treatment effect was found in the CA3 region ($F_{(2,22)}=4.583$, $P < 0.05$). Pairwise comparisons (LSD test), however, only revealed a trend towards a higher expression of 5-HT\textsubscript{1A} receptor mRNA in LAL mice housed opposite a SAL male ($P = 0.053$ vs. control). A line effect was observed in the CA1 region ($F_{(1,22)}=11.187$, $P < 0.005$), CA3 region ($F_{(1,22)}=23.269$, $P < 0.001$), and dentate gyrus ($F_{(1,22)}=28.184$, $P < 0.001$) of the hippocampus. Control LAL mice showed a significantly lower mRNA expression of the 5-HT\textsubscript{1A} receptor than control SAL mice in the CA3 region ($P < 0.001$), and dentate gyrus ($P < 0.005$). LAL mice housed opposite a SAL male showed a significantly lower 5-HT\textsubscript{1A} receptor mRNA expression than SAL mice housed opposite a SAL male in CA1 ($P < 0.005$), CA3 ($P < 0.05$), and dentate gyrus ($P < 0.01$). A significant treatment $\times$ line interaction was found in the CA1 region ($F_{(2,22)}=4.809$, $P < 0.05$). Here, 5-HT\textsubscript{1A} receptor mRNA was significantly lower in LAL mice housed opposite a SAL male ($P < 0.05$ vs. LAL control).

Summary of in situ hybridisation data

LAL mice living opposite a SAL male showed a significant increase in CRH mRNA in the PVN and in GR mRNA in the dentate gyrus, and a significant decrease in 5-HT\textsubscript{1A} receptor mRNA in hippocampal CA1 neurons. Line differences
were observed for the expression of hippocampal 5-HT$_{1A}$ receptor mRNA which was significantly lower in LAL mice.

**Table 1.** Effect of sensory contact with a SAL opponent for 5 days in LAL and SAL mice on the mRNA expression (in arbitrary units) of CRH and GR in the PVN and of GR and MR in the CA1 and dentate gyrus (DG) subregions of the hippocampus.

<table>
<thead>
<tr>
<th>Line</th>
<th>Treatment</th>
<th>CRH PVN</th>
<th>CRH CA1</th>
<th>CRH DG</th>
<th>GR PVN</th>
<th>GR CA1</th>
<th>GR DG</th>
<th>MR CA1</th>
<th>MR DG</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAL</td>
<td>control</td>
<td>67.5 ± 7.3</td>
<td>61.6 ± 1.9</td>
<td>49.3 ± 1.9</td>
<td>39.2 ± 1.2</td>
<td>77.5 ± 5.7</td>
<td>82.6 ± 5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAL</td>
<td>SAL opp</td>
<td>94.6 ± 7.5*</td>
<td>58.6 ± 1.8</td>
<td>50.0 ± 1.9</td>
<td>47.8 ± 1.8</td>
<td>82.7 ± 1.3</td>
<td>92.9 ± 4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAL</td>
<td>control</td>
<td>66.4 ± 4.2</td>
<td>54.6 ± 3.2</td>
<td>48.1 ± 2.4</td>
<td>41.9 ± 2.5</td>
<td>77.6 ± 4.6</td>
<td>84.9 ± 4.4</td>
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<td></td>
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<tr>
<td>SAL</td>
<td>SAL opp</td>
<td>82.9 ± 3.3b</td>
<td>56.6 ± 5.6</td>
<td>49.0 ± 0.7</td>
<td>44.2 ± 1.3</td>
<td>82.5 ± 4.1</td>
<td>89.4 ± 3.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* $P < 0.01$ vs. LAL control, \(b\) $P = 0.078$ vs. SAL control, \(c\) $P < 0.01$ vs. LAL control, pairwise comparisons (LSD) following ANOVA.

**Table 2.** Effect of sensory contact with a SAL opponent for 5 days in LAL and SAL mice on 5-HT$_{1A}$ receptor mRNA expression (arbitrary units) in CA1, CA3 and dentate gyrus subregions of the hippocampus.

<table>
<thead>
<tr>
<th>Line</th>
<th>Treatment</th>
<th>CA1 region</th>
<th>CA3 region</th>
<th>Dentate gyrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAL</td>
<td>control</td>
<td>104.2 ± 4.2</td>
<td>23.4 ± 1.9c</td>
<td>15.4 ± 1.4c</td>
</tr>
<tr>
<td>LAL</td>
<td>SAL opp</td>
<td>91.2 ± 5.4ac</td>
<td>30.9 ± 3.2bc</td>
<td>21.5 ± 2.7c</td>
</tr>
<tr>
<td>SAL</td>
<td>control</td>
<td>109.1 ± 4.7</td>
<td>37.9 ± 3.2</td>
<td>34.5 ± 4.9</td>
</tr>
<tr>
<td>SAL</td>
<td>SAL opp</td>
<td>115.0 ± 1.8</td>
<td>41.5 ± 2.1</td>
<td>37.6 ± 2.1</td>
</tr>
</tbody>
</table>

* $P < 0.05$ vs. LAL control, \(b\) $P = 0.053$ vs. LAL control, \(c\) $P$ at least < 0.05 vs. SAL, pairwise comparisons (LSD) following ANOVA

**Experiment 2: Effect of type of opponent in the sensory contact model**

**Body weight (Fig. 5)**

Body weight was measured daily, starting just before the mice were placed in the partition cage (day 1) till day 5. Body weight at day 1 was set at 100%. Repeated measures ANOVA revealed a main effect for treatment \((F_{(3,52)} = 18.781, P < 0.001)\) and a time × treatment interaction \((F_{(12,208)} = 8.152, P < 0.001)\). All mice housed in the partition cage showed a significant decrease in relative body weight from day 2 till day 5 \((P$ at least < 0.05 vs. control, Fig. 5A,B). However, mice
housed opposite a LAL male showed less body weight loss than single housed mice at day 2 (only LAL, \( P < 0.05 \)) and mice housed opposite a SAL male at day 2 (only LAL, \( P < 0.05 \)) and day 3 (both mouse lines, \( P < 0.05 \), Fig. 5A,B).

![Graph A](image1.png)  
**Fig. 5.** Effect of type of opponent in sensory contact model on relative body weight of LAL (A) and SAL (B) mice. At day 1, mice were housed in the partition cage either single (single), opposite a LAL male (LAL opp) or opposite a SAL male (SAL opp) or were put in a novel cage (control). Body weight at day 1 was set at 100% for each mouse. * \( P \) at least < 0.05 vs. control, # \( P < 0.05 \) vs. LAL opp, pairwise comparisons (LSD test) following repeated measures ANOVA.

Corticosterone (Fig. 6A,B)  
Plasma corticosterone was measured by obtaining blood (by a tail cut at day 2 and by decapitation at day 5) between 0800 and 0900 (4½-3½ h before the dark phase). A main line effect was found (\( F_{(3,50)} = 5.943, P < 0.005 \)). In addition, a time \( \times \) line interaction (\( F_{(1,50)} = 12.034, P < 0.005 \)) and a time \( \times \) treatment \( \times \) line interaction (\( F_{(3,50)} = 3.086, P < 0.05 \)) was found. Control LAL mice had lower corticosterone concentrations than control SAL mice at day 2 (\( P < 0.01 \), Fig. 6A). Single housed LAL mice showed corticosterone concentrations that were significantly lower at day 2 (\( P < 0.05 \), Fig. 6A) but higher at day 5 (\( P < 0.05 \), Fig. 6B) compared to single housed SAL mice. LAL mice housed opposite a SAL male showed significantly higher corticosterone concentrations at day 2 compared to all other LAL groups (\( P < \) at least 0.05, Fig. 6A) and at both days compared to SAL mice housed opposite a SAL male (\( P < 0.01 \), Fig. 6A,B). At day 5 all LAL mice housed in the partition cage showed significantly higher corticosterone concentrations compared to control LAL mice (\( P < 0.005 \), Fig. 6B). Within the SAL line, no significant treatment effect was found (Fig. 6A,B).
ACTH (Fig. 6C)

Plasma ACTH concentrations were measured at day 5 by obtaining blood by decapitation, between 0800 and 0900 (4½-3½ h before the dark phase). Univariate ANOVA showed a significant treatment \( F(3,67) = 4.388, P < 0.01 \) and line effect \( F(1,50) = 11.326, P < 0.005 \). Only SAL mice housed opposite a LAL male showed significantly higher ACTH concentrations compared to all other SAL groups \( P \) at least < 0.005, Fig. 6C) and compared to LAL mice housed opposite a LAL male \( P < 0.05 \), Fig. 6C).

![Corticosterone Day 2](image)

![Corticosterone Day 5](image)

![ACTH Day 5](image)

Fig. 6. Effect of type of opponent in sensory contact model on plasma corticosterone concentrations at day 2 (A) and day 5 (B) and ACTH concentrations at day 5 (C) in LAL and SAL mice. * \( P \) at least < 0.05 vs. all other LAL groups (A), vs. control (B), vs. all other SAL groups (C), # \( P \) at least < 0.05 vs. SAL, pairwise comparisons (LSD test) following univariate ANOVA.

Organ weights (Fig. 7)

Several organs were weighed as possible peripheral indicators of altered HPA axis functioning. All organ weights were corrected for body weight at day 5.

Thymus weight (Fig. 7A). A significant treatment \( F(3,67) = 12.423, P < 0.001 \) and line \( F(1,52) = 6.839, P < 0.05 \) effect was found. All mice housed in the partition cage, except for single housed SAL mice, showed a significant reduction in relative thymus weight \( P \) at least < 0.01 vs. control groups). Compared to SAL, control LAL mice and LAL mice housed opposite a SAL male had higher relative thymus weight \( P < 0.05 \).

Adrenal weight (Fig. 7B). A significant line effect \( F(1,52) = 6.614, P < 0.05 \) and treatment \( \times \) line interaction \( F(3,52) = 4.093, P < 0.05 \) was found. LAL mice housed opposite a SAL male showed significantly higher adrenal weights \( P \leq 0.01 \) vs. all other LAL groups). SAL mice housed single showed higher adrenal weights.
than LAL mice housed single \((P < 0.05)\). SAL mice housed opposite a LAL male showed higher adrenal weights than SAL control \((P < 0.01)\) and than LAL mice housed opposite a LAL male \((P < 0.005)\).

**Seminal vesicle weight (Fig. 7C).** A significant treatment \((F_{(3,52)} = 4.511, P < 0.01)\) and line \((F_{(1,52)} = 16.138, P < 0.001)\) effect was found. LAL mice housed opposite a SAL male showed significantly lower seminal vesicle weights \((P < 0.005\) vs. LAL control, \(P < 0.05\) vs. single housed LAL mice). All LAL groups housed in the partition cage showed significantly lower seminal vesicle weights than all SAL groups housed in the partition cage \((P < 0.05)\).

**Spleen and testes.** No treatment or line effects were found (data not shown).

**A Thymus**

**B Adrenals**

**C Seminal vesicles**

Fig. 7. Effect of type of opponent in sensory contact model on relative organ weights [organ weight (mg)/body weight (g)] of LAL and SAL mice after 5 days in the partition cage. (A) Relative thymus weight. (B) Relative adrenal weight. (C) Relative seminal vesicle weight. * \(P\) at least < 0.05 vs. control, vs. all other LAL groups (B), **\(P\) at least < 0.05 vs. SAL, pairwise comparisons (LSD test) following univariate ANOVA.

**Behaviour in the partition cage**

Behaviour of mice in the partition cage was scanned for 13 times per session of one hour during the late light phase (of day 1, 2 and 3) and during the early dark phase (of day 1, 2 and 4).

**Frequency at partition zone.**

No treatment or line effect was found (data not shown).

**Inactivity (Fig. 8).**

Overall effects were found for treatment \((F_{(2,33)} = 5.835, P < 0.01)\), line \((F_{(1,33)} = 43.703, P < 0.001)\), and treatment \(x\) line \((F_{(2,33)} = 6.278, P < 0.01)\). Furthermore, a significant time \(x\) line interaction \((F_{(5,165)} = 3.126, P < 0.05)\) was found. LAL mice
housed opposite a LAL male were significantly more inactive than single housed LAL mice ($P$ at least $< 0.05$ during the last three days, Fig. 9A) and than SAL mice housed opposite a LAL male ($P$ at least $< 0.05$ at all time points). Furthermore, LAL mice housed opposite a SAL male were significantly more inactive than single housed LAL mice ($P$ at least $< 0.05$ during the last three days, Fig. 9A), than LAL mice housed opposite a LAL male ($P < 0.01$ dark phase at day 1, Fig. 9A) and than SAL mice housed opposite a SAL male ($P$ at least $< 0.05$, during all dark phases and light phase at day 3).

**Fig. 8.** Frequency of inactivity of LAL (A) and SAL (B) mice in the partition cage. Behaviour was measured by a scanning procedure in which each mouse was scanned every 5 min for 1 h, resulting in a maximum score of 13 per session. This procedure was carried out during the late light phase (1100 till 1200) and early dark phase (1530 till 1630) at several days. $^*$ $P$ at least $< 0.05$ vs. single housed mice and vs. mice housed opposite a LAL male at day 1, pairwise comparisons (LSD test) following repeated measures ANOVA.

*Exploration (data not shown).*

Overall effects were found for treatment ($F_{(2,33)} = 10.893$, $P < 0.001$), line ($F_{(1,33)} = 26.734$, $P < 0.001$), and treatment × line ($F_{(2,33)} = 3.466$, $P < 0.05$). Furthermore, a significant time × line interaction was found ($F_{(5,165)} = 3.410$, $P < 0.01$). Single housed LAL mice showed significantly lower exploration activity during the dark phase at day 4 than single housed SAL mice ($P < 0.05$). LAL mice housed opposite a LAL male showed significantly lower exploration behaviour than single housed LAL mice ($P < 0.05$, during the dark phase at day 2) and than SAL mice housed opposite a LAL male (during the light phase at day 1 and during the last three days, $P$ at least $< 0.05$). LAL mice housed opposite a SAL male
showed significantly less exploration behaviour than single housed LAL mice ($P < 0.05$, all dark phases and during the light phase at day 3), than LAL mice housed opposite a LAL male ($P < 0.005$, dark phase at day 1) and than SAL mice housed opposite a SAL male ($P < 0.01$, during all dark phases).

Grooming (data not shown).

Small but significant overall effects were found for treatment ($F_{(2,33)} = 4.419$, $P < 0.05$) and line ($F_{(1,33)} = 4.952$, $P < 0.05$). Grooming behaviour was significantly higher in SAL mice housed opposite a SAL male than in SAL mice housed opposite a LAL male (during the dark phase at day 1, $P < 0.05$) and than in LAL mice housed opposite a SAL male (during the light phase of day 3, $P < 0.01$).

Eating/drinking (data not shown).

An overall effect was found for treatment ($F_{(2,33)} = 4.995$, $P < 0.05$). A small but significant time × treatment interaction ($F_{(10,165)} = 1.987$, $P < 0.05$) and time × treatment × line interaction was found ($F_{(5,165)} = 1.973$, $P < 0.05$). Single housed LAL mice showed more eating/drinking behaviour during dark phase of day 2 ($P < 0.005$ vs. all other LAL groups; $P < 0.05$ vs. SAL mice). Compared to SAL mice, LAL mice housed opposite a LAL male showed less eating/drinking behaviour ($P < 0.05$, during the dark phase at day 1) while LAL mice housed opposite a SAL male showed more eating/drinking behaviour ($P < 0.05$, at day 3).

Social interaction (data not shown).

Overall effects were found for treatment ($F_{(1,23)} = 9.129$, $P < 0.01$), line ($F_{(1,23)} = 38.630$, $P < 0.001$), and treatment × line ($F_{(1,33)} = 7.211$, $P < 0.05$). Furthermore, a significant line effect was found ($F_{(5,115)} = 4.855$, $P < 0.001$). Social interaction with the opponent was found to be significantly higher in SAL mice housed opposite a SAL male than in SAL mice housed opposite a LAL male (at day 1, $P < 0.05$; dark phase at day 2, $P < 0.005$) and than in LAL mice housed opposite a SAL male (at day 1 and during the dark phase at day 2, $P < 0.001$).

Summary of behaviour

A consistent treatment and line effect was found for inactivity behaviour (and along with this for exploration behaviour as well). Inactivity was higher in LAL mice housed opposite a LAL or SAL male compared to similar SAL groups and single housed LAL mice at several time points. Grooming and eating/drinking behaviour did not show a consistent change. During the first two days in the partition cage, social interaction was the highest between two SAL males.
Discussion

In a previous experiment, we showed that living in sensory (except for tactile) contact with a SAL male for 25 days induced a distinctly different response in long attack latency (LAL) males compared to short attack latency (SAL) males (Veenema et al., 2003b). In the present study, it was shown that this difference in the response of LAL and SAL mice to the daily sensory contact stressor was already present after 5 days. At this time point, only LAL mice showed pronounced changes that were induced by the stressor. These changes included elevated corticosterone levels, adrenal hypertrophy, low seminal vesicle weight and changes in the mRNA expression of CRH in the PVN, and GR and 5-HT1A receptor in the hippocampus. It was further demonstrated that the magnitude of the changes depended on the type of opponent (i.e. single housed vs. LAL opponent vs. SAL opponent). Housing mice single (isolation) already induced activation of the stress system in both mouse lines, although this was more pronounced in LAL mice. The highest response, however, was observed in LAL and SAL males living opposite a male of the other line. This was indicated in LAL mice by lower body weight, higher corticosterone levels, adrenal hypertrophy, and lower seminal vesicle mass, and in SAL mice by higher ACTH levels and adrenal hypertrophy compared to the other treatments.

Effect of sensory contact stress with a SAL male for 5 days

Body weight was significantly decreased in LAL and SAL mice living in sensory contact with a SAL male for 5 days, which is consistent with previous findings (Veenema et al., 2003b). Yet, corticosterone levels were significantly higher in LAL mice living opposite a SAL male compared to their SAL counterparts. In addition, adrenal hypertrophy and lower seminal vesicle mass (peripheral indicators of chronic stress) were only observed in LAL mice. This indicates higher severity of the stressor in LAL mice. In fact, the absence of stress levels of corticosterone at day 5 in SAL mice suggests that the sensory contact stressor had only transient effects. This would be in agreement with our previous finding in which no stress symptoms were observed in SAL mice when living opposite a SAL male for 25 days (Veenema et al., 2003b).

Although corticosterone levels were still elevated at day 5 in LAL mice housed opposite a SAL male, this was not associated with elevated ACTH levels. Similar discrepancies between ACTH and corticosterone in response to stressors have been reported by others (Gomez et al., 1996), and this may indicate a change in adrenocortical sensitivity to ACTH in LAL mice. The line differences found for ACTH (lower in LAL mice) and thymus weight (higher in LAL mice) are likely to be a trait characteristic as similar findings were reported under stress-free conditions (Veenema et al., 2003a). Collectively, these peripheral data indicate that
living opposite a SAL male for 5 days induced a different effect in LAL and SAL mice, showing a higher stressor susceptibility in LAL mice.

In support of the data on peripheral parameters, living opposite a SAL male for 5 days induced several central changes in LAL mice but not in SAL mice. CRH mRNA in hypothalamus and GR mRNA in the dentate gyrus were up-regulated, while 5-HT\textsubscript{1A} receptor mRNA in CA1 hippocampal neurons was down-regulated and hypothalamic GR mRNA and hippocampal MR mRNA were not affected. This is in sharp contrast with our previous finding in LAL mice after 25 days of continuously living opposite a SAL male (Veenema et al., 2003b). At that time point, elevated plasma corticosterone levels in LAL mice were associated with a significant decrease in hippocampal MR mRNA (Veenema et al., 2003b). A decrease in MR has been hypothesized to be one of the hallmarks of depressive illness because of its down-regulation by chronic stress and recovery or up-regulation after treatment with anti-depressant drugs (De Kloet, 1991; Reul et al., 1993, 2000; De Kloet et al., 1998; Lopez et al., 1998). The rise in hypothalamic CRH mRNA after 5 days of continuous exposure to the sensory contact stressor may have contributed to the development of the HPA axis abnormalities seen after 25 days. Hyperactivity of CRH neurons within the hypothalamus has been associated with the development of HPA hyperactivity seen in human depression (Keck and Holsboer, 2001). The region-specific up-regulation in GR mRNA in association with high corticosterone levels in LAL mice is not easily explained. GR activation in particular the hippocampus might, however, have a positive rather than a negative feedback effect on the HPA axis (van Haarst et al., 1997), but before suggesting such a mechanism here, more research is needed.

The stress-induced down-regulation of 5-HT\textsubscript{1A} receptor transcript in CA1 hippocampal neurons in LAL mice likely was a temporal phenomenon, since no change in 5-HT\textsubscript{1A} receptor protein was found after 25 days of living opposite a SAL male (Veenema et al., 2003b). These data clearly show that within LAL mice, central markers of the stress system were susceptible to changes along with the duration of the sensory contact stressor. Time-dependent central HPA changes have also been reported in other stress models (Liberzon et al., 1999; Buwalda et al., 2001). Whether these central changes seen in LAL mice after 5 days of sensory contact stress triggers the pattern of HPA abnormalities seen after 25 days, remains to be elucidated.

**Effect of type of opponent in sensory contact model**

By including two groups of mice (single housed and living opposite a LAL male) to the sensory contact model, it could be demonstrated that isolation (single housed mice) already induced activation of the stress system. This was shown by a reduction in body weight in both mouse lines and thymus involution and elevated corticosterone levels at day 5 in LAL mice. However, living opposite a SAL male
induced in LAL mice at day 2 and 3 a significantly larger body weight loss than living opposite a LAL male. This larger body weight loss in LAL mice living opposite a SAL male was further associated with significantly higher corticosterone levels at day 2 and adrenal hypertrophy compared to all other LAL groups. This indicates that in LAL mice, the presence of a SAL male induced the highest stress response.

Interestingly, within all SAL groups, corticosterone levels at day 2 were found to be higher than baseline levels (baseline: 16.3 ± 3.3 µg/dL, see Veenema et al., 2003a). These high levels may have been induced because all mice (including the control groups) were put in a novel cage at the start of the experiment. This novel environment can be considered as a stressor particularly in the aggressive SAL mice as they had to re-establish their territory. Indeed, elevated circulating corticosterone concentrations have been found in male laboratory mice that show territorial aggression to achieve a dominant position compared to subdominant passive mice (Haemisch and Gartner, 1997). In SAL mice, this rise in corticosterone was, however, transient as corticosterone levels at day 5 were comparable to baseline levels, except for SAL mice housed opposite a LAL male. This latter group showed relatively high levels of corticosterone, adrenal hypertrophy and elevated ACTH concentrations, suggesting that in SAL mice the presence of a LAL male was still stressful after 5 days.

The lower seminal vesicle mass in all LAL mice housed in the partition cage compared to SAL mice, might indicate higher susceptibility to stress-induced inhibition of the reproductive axis in LAL mice, which could have been induced by increased glucocorticoid secretion (Rabin et al., 1988; Rivier and Rivest, 1991). Collectively, the data demonstrate a higher susceptibility of LAL mice living opposite a SAL male than of SAL mice living opposite a LAL male.

By scanning the behaviour of mice in the partition cage, it was shown that both mouse lines were highly active during the first day in the partition cage, while at the other days a clear circadian rhythm was established with lower activity during the light phase and higher activity during the dark phase. This novelty-induced activity behaviour at day 1 may have contributed to the observed reduction in body weight. Although a preference for the partition area was not different between the mice groups, social interaction was the highest between two SAL males. This may show that the threshold to fight was the lowest between two aggressive males. Living in sensory contact with either a LAL or SAL male induced a significant increase in inactivity behaviour in LAL mice. A decrease in motor activity was also reported in subordinate tree shrews living in sensory contact with a dominant male (Fuchs et al., 1996; Kramer et al., 1999). These longer periods of behavioural inactivity may reflect a change in the emotional state of LAL mice associated with increased anxiety and/or decreased risk assessment behaviour.
In conclusion, LAL and SAL mice responded differently to a SAL male in the 5 days sensory contact model. The LAL mice showed after 5 days pronounced HPA changes, that were however very different from the effects observed after 25 days of continuous exposure to the sensory contact stressor. In SAL mice, 5 days sensory contact evoked only transient effects in the HPA axis, while the decreased body and thymic weight persisted. Although isolation of the mouse already induced many stress symptoms, most pronounced effects were observed when SAL and in particular LAL mice were housed opposite a male of the other line. This indicates that stressor susceptibility in LAL and SAL mice depends on the type of opponent in the sensory contact model.

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References


