Chapter 6

Repeated rat exposure inhibits the circadian activity patterns of C57BL/6J mice in the home cage

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Abstract

Exposing male C57BL/6J mice repeatedly, in an unpredictable and uncontrollable fashion to rats, alters their cognitive performance and the neuroendocrine stress response, weeks to months after the rat stress. Continuous observation of the behavioral activity of male C57BL/6J mice in their home cage before (baseline) and after rat exposure could reveal if repeated rat exposure leads to changes in circadian activity patterns, which is a key feature of chronic stress and stress-related disorders in humans.

Rat stress (1) decreased exploratory and foraging activity as characterized by increased time spent in the shelter and less time in the open area; (2) reduced sucrose consumption and inhibited the development of sucrose preference, suggesting changes in the reward system and (3) the exploration pattern in a novel environment included more behavioral perseverations, but no change in general locomotor activity. Comparison to baseline activity pattern, i.e. before any intervention, revealed that already the control procedure to rat exposure (spending the same amount of time in another cage) disrupted the organization of behavioral activity patterns, albeit to a different and lesser degree than observed in rat stressed mice.

While only the longitudinal design of the study allowed detecting these dynamic patterns of circadian activities, the distinct behavioral changes in foraging and explorative activities support our notion that repeated rat exposure might serve as mouse model of chronic stress.

Introduction

Chronic stress, specifically a dysregulation of the glucocorticoid system, is thought to be a precipitating factor in the etiology of affective disorders (de Kloet et al. 2005). These disorders share several characteristics: emotional changes related to approach/avoidance behavior, loss of interest or pleasure in daily activities, impairment of cognitive functions, reduced motor activity and alterations in the circadian pattern of physiological, neuroendocrine and behavioral responses (Endo and Shiraki 2000; Volkers et al. 2002; Keller et al. 2006). The effects of chronic stress in animal models are mainly assessed in short-lasting test-situations involving additional novelty stress. Surprisingly little is known about the consequences of stress on the daily organization of behavior in a familiar environment where the animal spends most of its time: the home cage. This will be the focus of the present study.

Whereas all kinds of stressors induce behavioral alterations and concomitant changes in stress system regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Endo and Shiraki 2000; Anisman and Matheson 2005), psychological stressors are ethologically relevant and resemble the kind of stress that is related to affective disorders in humans (Calvo-Torrent et al. 1999; Apfelbach et al. 2005; Beekman et al. 2005). Central features of chronic psychological stressors in humans are repeated, unpredictable and uncontrollable exposure to (or imagination of) threatening situations. Animal models make use of confrontations with territorial conspecifics and exposure to predators with or without physical confrontation (Apfelbach et al. 2005). Interestingly, already sensory stimuli (visual, auditory and olfactory) appear to be sufficient to activate the stress system associated with the release of glucocorticoids (Blanchard et al. 1998; Diamond et al. 1999; Linthorst et al. 2000a; Beekman et al. 2005). In rodents, the behavioral effect of predator exposure is manifested as increased anxiety-like behavior, risk-assessment in novel environments and learning and memory impairments (Calvo-Torrent et al. 1999; Grootendorst et al. 2001a; Grootendorst et al. 2001b; Adamec et al. 2004; Diamond et al. 2006). The amplitude of circadian locomotor activity and food-intake in rats decreases after social conflict, “chronic mild stress” or electric shocks (Willner 1984; Desan et al. 1988; Stewart et al. 1990; Gorka et al. 1996; Meerlo et al. 1999). Resident/intruder pairs of mice living in continuous sensory contact and daily physically interaction reveal a variety of changes in behavior, autonomic and immune functions, HPA responses, brain cytokine expression and cardiac histology (Bartolomucci et al. 2005). To our knowledge, detailed patterns of activity in the home cage before, during and after a psychological stressor, without physical contact, have not been described in mice.

Previous studies have shown that, long term automatic recordings of the location of the mice in their home cage allows detailed observations on dynamic changes in locomotor activity over days, with minimal human intervention (de Visser et al. 2005; de Visser et al. 2006). In addition, the home cage is a familiar environment in which no specific behavior of the animal is elicited or challenged in some way. Subtle changes in spontaneous behaviors under base line conditions may reveal themselves more easily in the home cage than under conditions where the animal is prompted to explore or face a strong challenge.

The aim of the present study was to investigate the daily behavioral organization of mice in the familiar environment of their home cage: before, during and after chronic 'rat stress' exposure, which took place in a novel environment.

Our chronic stress model makes use of the observation that mice and rats avoid each other in nature. Indeed, repeated, unpredictable and uncontrollable exposure of mice to rats strongly activates the HPA axis (Grootendorst et al. 2001a; Grootendorst et al. 2001b). To control the effect of rat exposure on HPA axis activation, blood plasma corticosterone concentrations were measured before and after the first rat exposure. Furthermore, we determined consumption of and preference for a sucrose solution as markers for altered consumatory behavior and anhedonic consequences of stress. To address behavioral changes to novelty, exploration patterns of mice in a novel environment (circular hole board) were assessed two days after the last rat exposure. Principal Component Analysis (PCA) was performed to determine the relationship between activity parameters in the home cage which may be indicative of underlying motivational systems.

Materials and Methods

Animals

Upon arrival at the animal facilities of Utrecht, male C57BL/6J mice (Janvier Bioservices, The Netherlands; n = 32; 8 weeks) were individually housed with food and water *ad libitum* in Macrolon Type II cages for one week. The room was temperature (19 - 21°C) and humidity (30 - 50%) controlled with a 12-12h light-dark cycle (lights on 0800-2000h). A shelter and nesting material (tissues, paper shreds) were provided. Thereafter, mice were housed individually in PhenoTyper cages. Male Long Evans hooded rats (Janvier, n = 8; 250 - 275g) were housed in pairs in a separate room, with food and water *ad libitum*. Experiments were approved by the Local Committee for Animal Health, Ethics

and Research of the Universities of Leiden and Utrecht. Animal care was conducted in accordance with the EC Council Directive of 24 November 1986 (86/609/EEC) and the Principles of laboratory animal care (NIH publication No. 86-23, revised 1985).

Experimental design

Figure 1 depicts an overview of the experimental schedule. Mice were housed for one week in Macrolon Type II cages (days -6 to 0). These cages were kept with the original bedding but without nesting material, for rat stress and control procedures. Automated registration of activity and location of the mice took place in the PhenoTyper home cages every day for 24h from days 1 to 19, and was interrupted only by experimental procedures. Mice were subjected to two conditions (n = 16/condition); (i) stress: exposure to rats during 2 weeks and (ii) control: placement into their first housing Macrolon Type II cages at similar times and duration as the rat stress condition. On day 6, blood samples were collected via tail incision before and directly after the first rat exposure. Sucrose solutions were available for 24h on days 5, 13 and 17. The exploration strategy of a

Day		Experimental manipulation
-6 – 0		Housing in Macrolon Type II cages
1	Housing in PhenoTyper	Baseline bodyweight
2		
3		
4		Baseline home cage activity and location
5		Baseline sucrose consumption/preference
6		Rat exposures 1 and 2
7		Rat exposures 3 and 4
8		Rat exposures 5 and 6
9		Rat exposure 7
10		Rat exposures 8 and 9
11		
12		
13		Sucrose consumption/preference
14		Rat exposure 10
15		
16		Rat exposure 11
17		Sucrose consumption/preference
18		Exploration on the circular hole board, 5 min
19		

Figure 1
Time line of the experiment. Data of home cage activities are presented from several days, shaded in gray (see Figures 4, 5 and 6). Black squares at day 6 indicate time of blood sampling for determination of corticosterone concentrations.

novel environment (the circular hole board), was assessed on day 18. Bodyweight was measured from the day of arrival until the end of the experiment on a daily basis.

Home cage behavior

Apparatus - Home cage behavior was automatically recorded by videotracking in specially designed cages for automated recordings (PhenoTyper®, Noldus Information Technology, Wageningen, The Netherlands, see Figure 2A). Each cage (30cm x 30cm x 35cm) was equipped with a feeding station and two drinking bottles. A shelter (10cm x 10cm x 5cm), bedding (sawdust) and nesting material (tissues) were provided. Hardware for videotracking is integrated in a unit on top of the cage, which also contains a built-in digital infrared-sensitive video camera and infrared lighting sources. The infrared sources provide a constant and even illumination of the cage to allow videotracking irrespective of light conditions in the experimental room.

Videotracking - EthoVision 3.1 (Noldus IT, The Netherlands) was used as videotracking software. Within EthoVision, we designed several zones per cage to extract behavioral measures of duration and frequencies of visits per zone (see Figure 2B): shelter, feeder, bottles 1 and 2. The open area covers the rest of the surface. The shelter was defined as a "hidden zone" allowing the program to distinguish the location of the mouse as being "in the shelter" or "on the shelter".

Videotracking was performed with the maximal sample rate of 12.5 samples/second. The system is programmed to score changes in the location of the centre of gravity of the mouse as "movement", only when the mouse moved at least with a velocity of 3.5cm/second, averaged over 12 samples. This excludes small movements of

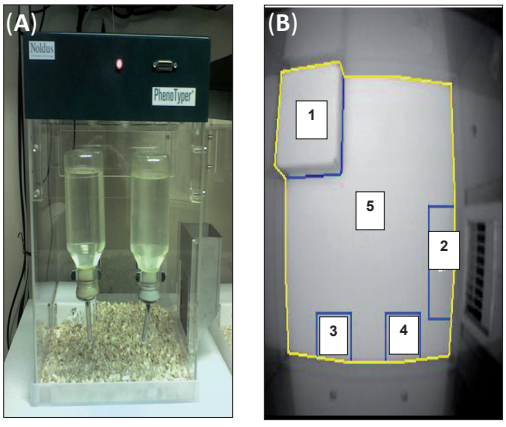

Figure 2
(A) Picture of the automated home cage observation system with two drinking bottles, shelter and feeding area. **(B)** Schematic overview of the different zones that were used for analysis of home cage behavior: 1=shelter; 2=feeder; 3=bottle 1; 4=bottle 2; 5=open area.

the animal caused by e.g., turning or moving around while staying in the same place. The number of stops was calculated using the frequency of 'non-movement' fragments, per unit distance movement. This yielded a measure that is independent of overall amount of activity (de Visser et al. 2006). Velocity was calculated only of 'movement' episodes, thus excluding periods of non-movement.

Dependent variables - Per zone, several parameters were calculated which were subjected to further analysis: duration (time spent in a specific zone), frequency (number of visits to and from a specific zone), cage floor movement (time spent moving in the open area in seconds), distance moved (distance travelled by the animal in the open area in cm), velocity (speed of moving in cm/s) and the number of stops (periods of non-movement per unit distance moved). All parameters were calculated in 1-hour intervals and subsequently summed for the 12-hours fragments of the dark and light period. For the nocturnal pattern of activity, we used the hourly values of the parameter cage floor movement during the dark period.

Rat stress paradigm

Exposure to a rat profoundly activates the Hypothalamic-Pituitary-Adrenal (HPA) axis of the mouse, resulting in elevated concentrations of corticosterone in brain lysate (Linthorst et al. 2000) and blood plasma (Grootendorst et al. 2001a). We have designed the "mouse-exposed to rat" procedure some years ago (Grootendorst et al. 2001a; Grootendorst et al. 2001b), taking into account central features of a stressor: unpredictability, uncontrollability. The protocol uses repeated exposure to rats (i) either daily or with a break of one or several days; (ii) varying the duration and time of the rat exposure: 1 or 2h; once or twice a day; (iii) each time placing different rats on top of the mouse cage – and thus avoiding physical contact that might involve pain.

Several times between days 6 - 16, mice were exposed to a rat (Figure 1). During the first week (days 6 - 10) of the rat stress paradigm, mice were exposed to rats on 5 consecutive days (one or two hours a day resulting in a total exposure time of nine hours). In the second week (days 13 - 16), two exposures took place: on Tuesday (1h) and Thursday (1h). On rat-exposure-days, mice were placed and transported in their first Macrolon Type II housing cages from the PhenoTyper to the adjacent rat stress room. One rat was placed on top of two mouse cages. Mice and rats were separated by a grid and could see, hear and smell, but not touch each other. Food and water was not available during rat exposure. The person who performed the rat stress procedure did not enter the housing room of the mice, as to avoid confrontation of control mice with the smell of rats. To keep time and duration of exposure unpredictable for the mice, the

exposures took place at different times during the light period, lasting one or two hours, either once or twice a day. The location of the cages and the combination of the rat and the mouse were changed *ad random*. Rat stress thus consisted of transportation and the exposure of mice to rats. Control mice were placed into their previous Macrolon Type II cage for the same duration as mice exposed to rats, but were not transported to another room. Thus, in both conditions, mice were removed from the PhenoTyper home cage. To asses the effect of rat exposure on arousal, mice were weighed before and directly after the last rat exposure of the day. Weight loss was taken as a measure of defecation and urination. Similar time points were used for control mice.

Sucrose consumption

Throughout the experiment two drinking bottles were fixed to the home cage. To determine if mouse preferred to drink from one of the two water bottles, the bottles were weighed on days 1 and 4. During sucrose consumption/preference testing, water from the least preferred bottle was replaced with a 5% sucrose solution. Consumption of water and sucrose was determined one day before (baseline; day 5), two days after the ninth rat exposure (day 13) and one day after the last rat exposure (day 17). The bottles were weighed before placement and after removal 24h later. Water and sucrose consumption is expressed in ml. The preference for sucrose was calculated as percentage of consumed sucrose solution of the total amount of water and sucrose consumption. After sucrose testing, both bottles contained water.

Blood sampling and corticosterone measurement

To determine the level of stress system activation in response to experimental procedures, blood was collected via tail incision (Dalm et al. 2005) before and after one hour of rat exposure (stress group) or placement in the Macrolon Type II home cage, on day 6. Blood was collected individually in capillaries (coated with potassium-EDTA, Sarstedt, Germany), kept on ice and centrifuged for 10 minutes with 13000 rpm at 4°C. Blood plasma was stored at –20°C. Plasma corticosterone was analyzed using a commercial available radio immunoassay kit ^{125}I-corticosterone (MP Biomedicals CA; USA; sensitivity 3ng/ml).

Circular hole board

Apparatus: A grey round plate (Plexiglass; 110cm diameter) with 12 holes (5cm diameter, 5cm deep), at equal distances from each other and at a distance of 10cm

from the rim of the hole to the rim of the plate, was situated 1m above the floor in a different experimental room. Light conditions on the surface of the board were 120lux. To distribute odor cues, the surface was cleaned with 1%HAc and the board was turned (randomly clock- and anticlockwise) before a mouse was tested.

At day 18, two days after the last rat exposure, mice were taken from their home cage, transported in a Macrolon Type II cage, picked from the cage at the base of the tail, and placed in a grey cylinder (PVC, 10cm diameter; 25cm high) that was located in the center of the circular hole board. After 10s the cylinder was lifted and the mouse could start to explore the board for 5 min. Immediately thereafter, the mouse was transported back to the PhenoTyper home cage. Behavior was recorded on videotape and analyzed by EthoVision Windows 3.1 (Noldus Information and Technology BV, Wageningen, The Netherlands). The image analysis system sampled the position of the mouse 12.5 samples/second. To calculate the distance moved, we set the system to score movement when the mouse moved at least with a velocity of 3.5cm/second, averaged over 12 samples. The following parameters related to general activity, exploratory strategies and possible anxiety-related behaviors were analyzed: distance walked (m) on the board and in specified zones: center, rim; velocity (cm/s), number of holes visited; sequence of hole visits (*serial*: more than two hole ins sequence; *perseveration*: repeatedly visiting the same hole or alternately visiting two neighbouring holes); latency (s) to leave the center; latency (s) to rim; time (s) in specified zones. The center is defined as a circle of 30cm diameter; hole area: a ring of 15cm with the holes in the middle; rim area: a ring of 4.5cm at the outer perimeter of the plate.

Additional experiment on sucrose consumption and novel cage in non-stressed mice

It has been shown that HPA axis activity and sucrose consumption are linked (Bell et al. 2000; Laugero et al. 2001). To assess if sucrose consumption itself might affect locomotor activity in the dark period, we performed an additional experiment. We used a separate set of male C57BL/6J mice (n = 16) to assess the effect of sucrose consumption on activity patterns in the home cage. Some mice were also exposed for two hours to their previous housing cage. Mice were initially housed individually in Macrolon Type II cages for one week (lights off from 1400 to 0200h; these cages, including the soiled bedding were kept and used for "novel cage" exposure later on). Thereafter, mice were housed in the PhenoTyper home cages for 5 days, and assigned to three groups: (1) *controls* (n=4) undisturbed from days 5 - 8; (2) *sucrose* (n=6) received a bottle with sucrose solution on day 5 at 0900h for 24h and not disturbed on days 6, 7 and 8; (3) *sucrose+NovelCage* (n

= 6) also received a bottle with sucrose solution on day 5 for 24h; on day 6, mice were placed in their previous Macrolon Type II cage (from 0900 - 1000h and 1300 - 1400h), and left undisturbed thereafter on days 7 and 8.

Statistical analysis

Home cage behavior: During the light period, activity was minimal and did not show any differences between groups or experimental days. Therefore, we limit the analysis to the dark period; i.e., the active period of mice, and selected the following experimental days (see Figure 1): day 4 (baseline), day 6 (after two exposures), day 10 (after nine exposures), day 14 (after 24hrs sucrose consumption and ten exposures) and day 19 (three days after the last exposure). Differences between control and stress groups for each day were determined using a *t*-test; within groups between days were tested by a paired samples *t*-test with a Bonferroni-correction for the number of comparisons. We selected to present the following parameters: time spent in the shelter, near the feeder and bottles and the distance moved in the open area, in Figure 4A-D. To test for differences between groups and days in the diurnal pattern of cage floor movement, repeated measures ANOVA was performed, using the within-subjects factors “hour”, for the 12 hours of the dark period, and “day”, for the four experimental days (6, 10, 14 and 17), and the between-subjects factor “group”, for control and stress groups. Post-hoc comparisons between days were done using repeated measures ANOVA with within-factors “hour” and “day”.

To investigate the interrelation of dependent variables measured in the home cage and to identify possible independent factors, we performed a Principal Component Analysis (PCA) with varimax rotation (Ferguson 1981; de Visser et al. 2006).Dependent variables that showed a loading >0.6 were regarded as being relevant for a specific factor. Factors with Eigenvalues >1 were retained for further analysis. To determine the factors, PCA was performed across individuals for baseline (day 4), thus before any manipulation had taken place. Then, based on this factor structure, factor scores were calculated for each animal on each day by multiplying the mean values of each 12-hour bin with the factor loadings to create new dependent variables. These new variables thus consist of the combined values of the dependent variables that belong to a given factor. These variables were further analyzed with a *t*-test to detect differences between control and stress groups and with a paired samples t-test to test for within-group differences between days. A Bonferroni-correction was used when multiple comparisons were made.

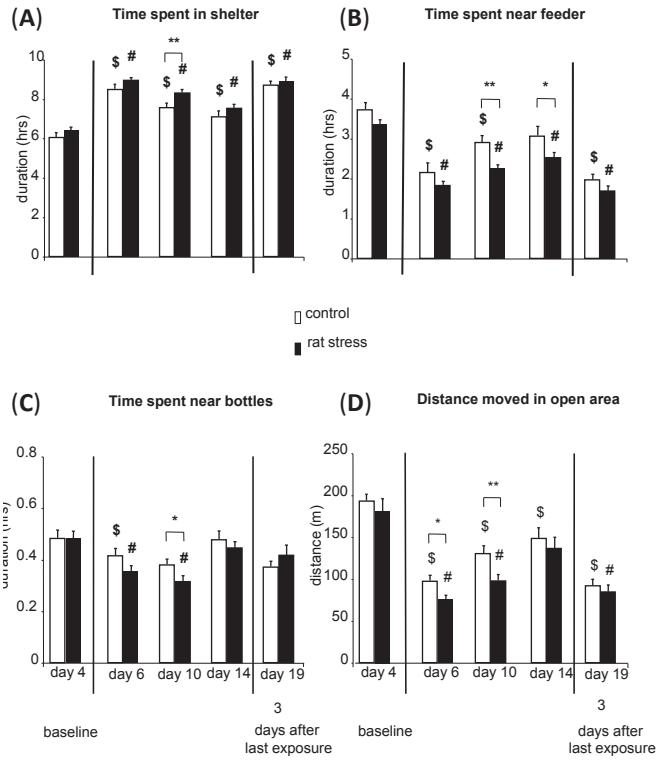

Figure 3

Home cage activity during the 12hrs lasting dark period: control (white bars) and stressed mice (black bars) on day 4 (baseline: before experimental manipulations start), day 6 (after two exposures), day 10 (after nine exposures), day 14 (after 24hrs sucrose consumption and ten exposures) and day 19 (three days after the last exposure and one day after circular hole board exploration). See Figure 1 for details on the experimental schedule. Home cage activity is represented by four dependent variables: **(A)** time in shelter, **(B)** time spent near the feeder, **(C)** time spent near the bottles and **(D)** distance moved in the open area. Data is presented as mean ± S.E.M. of the 12hrs dark period. Symbols indicate significant differences from baseline for control ($^{\$}$) and rat stress ($^{\#}$): paired samples t-test with Bonferroni correction $p < 0.05$; asterisks indicate significant differences between groups per experimental day: * $p < 0.05$, ** $p < 0.01$ (t-test).

Data on bodyweight, blood plasma corticosterone, sucrose consumption and preference, are presented as mean ± S.E.M.; tested using repeated measures ANOVA, and post-hoc t-test when appropriate. Depending on the normality score obtained with Kolmogorov-Smirnov, parameters of circular hole board behavior were compared using

one way ANOVA or Mann-Whitney test. Data is presented as mean ± S.E.M. Significance was accepted at $p < 0.05$.

Results

Home cage behavior

Mice were repeatedly exposed to rats (rat stress group) or placed in their previous housing cage in the same time schedule (control group). Rat stress had a distinctive influence on behavior of mice in the home cage (Figure 3A-D). These mice were less active than controls during the dark period. They spent less time at the feeder and the bottles and moved less (days 10 and 14, T-test, $p < 0.05$), but spent significantly more time in the shelter (day 10: T-test, $p < 0.05$). Three days after termination of the stress procedure, home cage behavior was comparable between control and stress groups for the parameters tested.

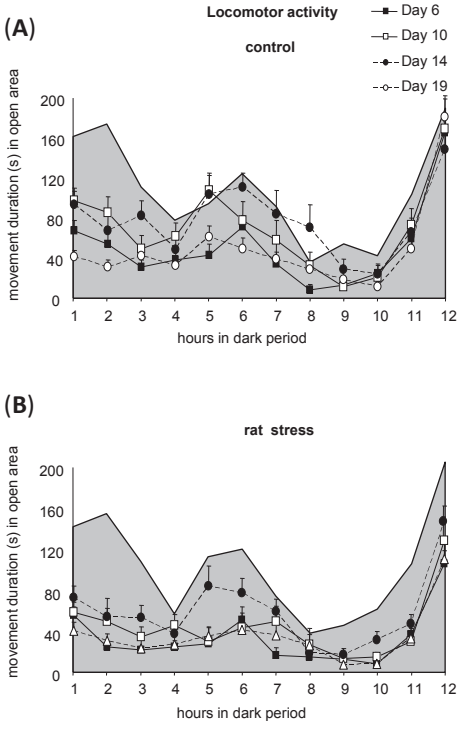

Figure 4

Hourly distribution of locomotor activity (cage floor movement), **(A)** control and **(B)** rat stress group, expressed as the time spent moving in the open area in seconds per hour (mean ± S.E.M.) during the dark period of day 4 (baseline), day 6 (after 2 exposures), day 10 (after 9 exposures), day 14 (after 10 exposures) and day 19 (three days after exposure). See Figure 1 for details on the experimental design. Shaded area represents mean activity on day 4.

Interestingly, also the control manipulation changed the level of nocturnal activity. Baseline measurements from day 4 allowed within-group analysis over days for control and stressed mice. Independent of group, significant differences from baseline were found for the time the mice spent in shelter (Figure 3A), near to the feeder (Figure 3B), near the drinking bottles (Figure 3C) and the distance moved in the open area (Figure 3D). For all days analyzed, activity in the open area as well as activity at the feeding and drinking places was lower compared to baseline (all days, paired samples T-test, $p < 0.05$). The drop in activity was largest in response to the first experimental manipulations in control and stressed groups (day 6), followed by a gradual increase towards baseline levels from days 10 to 14. Notably, three days after termination of the stress procedure on day 19, activity was comparable to levels observed on day 6: again, more time in shelter, less near the feeder and bottle area and reduced locomotion.

Nocturnal pattern of cage floor movement

The nocturnal pattern of cage floor movement is presented per hour for control (Figure 4A) and stressed mice (Figure 4B). As activity during the light period was minimal and did not show any group difference, it was decided to exclude these data from Figure 4A-B. Characteristic for the nocturnal pattern at baseline are the peaks in activity at the beginning and end of the dark period. Compared to baseline, both groups showed a significant change in the activity pattern (repeated measures ANOVA, interaction between within-subjects factors “day” and “hour”, $F_{(44,100)}=2.471$, $p = 0.003$). Post-hoc analysis revealed significant differences from baseline for all days (day 6, 10, 14 and 19; $p < 0.01$). The difference was most pronounced during the first 3 hours of the dark period, expressed by the markedly reduced peak during and after the experimental manipulations.

Principal Component Analysis of home cage behavior

The interrelation of variables measured in the home cage is represented by three factors, extracted by Principal Component Analysis (Table 1). The factors together accounted for 70.59% of the variance. Factor 1 loads positively on variables that indicate exploration, like time spent on the shelter and distance moved in the open area. Factor 1 is labelled “exploratory activity”. Factor 2 consists of variables that indicate the activity directed towards the feeding and drinking areas, like time spent near the feeder and the bottles: Factor 2 is labelled “foraging activity”. The third factor consists of variables like time spent in the open area, velocity and the number of stops suggesting a specific aspect of

Table 1: Principal Component Analysis

Variable	Factor 1	Factor 2	Factor 3
In shelter (frequency)	.691		
On shelter (frequency)	.791		
On shelter (duration)	.819		
Distance moved in open area	.920		
Cage floor movement	.881		
In shelter (duration)		-.898	
Feeder (duration)		.841	
Bottles (frequency)		.654	
Bottles (duration)		.775	
Open area (duration)			.690
Velocity			-.893
Number of stops			.619
% of variance explained	*31.50*	*22.90*	*16.19*

movement in the open area that is independent of general activity: Factor 3 is labelled "velocity/stops".

To investigate the effects of the stress paradigm on the individual scores of each of the factors, the factor scores of each mouse were calculated for the dark period of days 6, 10, 14 and 19. Figure 5 presents the data for Factors 1 and 2; Factor 3: "velocity/stops" was comparable between groups (data not shown).

Factor 1: "exploratory activity" was significantly decreased with respect to baseline on days 4 and 19 in control and stressed mice (paired samples T-test, $p < 0.05$). Stressed mice showed a further decrease compared to control mice during the stress procedure (days 6: t = 2.399, df = 30, $p = 0.023$; day 10: t = 2.355, df = 30, $p = 0.025$), but not after termination of the stress procedure (day 19). For Factor 2:"foraging activity", a decrease was found on all experimental days compared to baseline, both in control and stressed mice (paired samples T-test, $p < 0.05$). Furthermore, stressed mice scored less than controls during the stress procedure (days 6: t = 2.355, df = 30, $p = 0.025$; day 10: t = 4.581, df = 30, $p < 0.001$; day 14: t = 2.233, df = 30, $p = 0.033$). After termination of the stress procedure (day 19) factor scores were comparable between control and stressed mice.

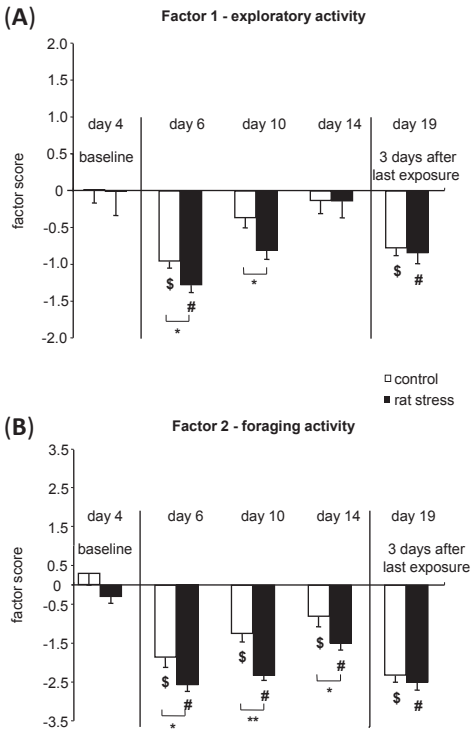

Figure 5

Home cage activity represented by two factors extracted from Principal Component Analysis (PCA); **(A)** Factor 1 indicates "exploratory activity"; **(B)** Factor 2 indicates "foraging activity". Factor scores were calculated for every mouse using factor loadings derived from PCA (see Table 1). Means per group for control (white bars) and rat stress (black bars) are presented for day 4 (baseline), day 6 (after two rat exposures), day 10 (after nine rat exposures), day 14 (after ten rat exposures) and day 19 (three days after the last rat exposure). See Figure 1 for details on the experimental design. Note that factor scores are relative measures and do not represent a specific measured variable. Data is presented as mean ± S.E.M. of the 12hrs dark period.

Symbol ($^{\$}$) indicate significant differences from baseline both groups (paired samples t-test with Bonferroni correction $p < 0.05$) asterisks (*) indicate significant differences between groups per experimental day: * $p < 0.05$, ** $p < 0.01$ (t-test).

In summary, removal from the home cage decreases both exploratory and foraging activity in control and stressed mice. However, there is a marked additive decreasing effect of rat exposure on exploratory activity and, more pronounced, on foraging activity.

Sucrose and water consumption and preference

Data are presented in Table 2. Fluid consumption on day 5, the day before rat stress began, was taken as baseline (100%): sucrose 12.7 ± 0.6ml and water 2.4 ± 0.1ml. The pattern of sucrose intake changed over time (time: $F_{(1,30)}$=47.646, p = 0.001) and was group dependent (group: $F_{(1,30)}$=24.896, p = 0.001). While sucrose intake was increased in both groups (paired samples T-test: $p < 0.05$) from day 13 to 17, this increase was significantly higher in controls than stressed mice, 42.2 vs. 17.7%, respectively. On both

Table 2: Sucrose and water consumption / preference in relation to baseline values. Baseline fluid intake: sucrose 12.7 ± 0.6 ml; water 2.4 ± 0.1 ml.

		Day 13 Two days after 9 exposures		Day 17 One day after 11 exposures	
	Fluid	% intake (ml)	preference (%)	% intake (ml)	preference (%)
Control	Sucrose	102.5 ± 4.8	88.7 ± 0.4	144.2 ± 5.3	91.3 ± 0.3
	Water	76.4 ± 3.7	11.2 ± 0.2	79.6 ± 4.9	8.7 ± 0.7
Rat stress	Sucrose	87.0 ± 5.8*	87.0 ± 0.8	104.7 ± 4.9*	87.2 ± 0.2*
	Water	68.1 ± 1.7*	12.9 ± 0.5	88.2 ± 3	11.9 ± 0.1

Data are expressed as mean ± S.E.M.

* $p < 0.05$ rat stress vs. controls.

testing days, stressed mice consumed less sucrose than controls (one way ANOVA, day 13: $F_{(1,30)}$=5.552, p = 0.025; day 17: $F_{(1,31)}$=38.188, p = 0.001). The pattern of water intake also changed over time (time: $F_{(1,30)}$=25.926, p = 0.001) and differed between groups (group: p = 0.001). Stressed mice had a significantly reduced water intake on day 13 (one way ANOVA, $F_{(1,31)}$=4.192, p = 0.049) which increased by 20% from day 13 to day 17 (paired samples T-test; t = -5.956, p = 0.001).

The pattern of sucrose preference, changed over time (time: $F_{(2,60)}$=26.996, p = 0.001) and was group dependent (group: $F_{(2,60)}$=13.753, p = 0.001). Stressed mice showed significantly less preference for sucrose than controls on day 17 (one way ANOVA, $F_{(1,32)}$=9.837, p = 0.004).

Novelty exploration - circular hole board

The behavioral pattern on the circular hole board differs significantly between the groups (MANOVA: $F_{(12,19)}$=3.521, p = 0.007; Table 3). Stressed mice had a longer latency to the first hole visit and hole dip (one way ANOVA, $F_{(1,31)}$=4.339, p = 0.049; Mann-Whitney, df=32; p = 0.035), while the total number of hole visits did not differ. Interestingly, stressed mice more often used perseveration strategies to visit the holes (one way ANOVA $F_{(1,31)}$=5.269, p = 0.029). Serial strategies, distance walked and velocity were comparable between groups.

Corticosterone response to rat exposure

Corticosterone concentrations before the first (rat) exposure on day 6 were comparable between groups. After rat exposure, higher corticosterone concentrations were

Table 3: Behavioral parameters indicative for exploration of the circular hole board.

Behavioral parameter	Control	Rat stress
Distance (m)	17.5 ± 0.8	17.4 ± 1.4
Speed of moving (cm/s)	5.8 ± 0.3	5.8 ± 0.5
Stops (number)	4.0 ± 0.2	4.5 ± 0.5
Latency first hole visit (s)	9.3 ± 1.0	13.6 ± 1.8*
Latency first hole dip (s)	21.2 ± 3.1	31.6 ± 4.3*
Total hole visits (number)	25.5 ± 1.8	24.4 ± 2.1
% serial	45.8 ± 3.8	57.2 ± 5.0
% perseveration	15.5 ± 3.5	24.4 ± 2.3*
Latency first rim dip (s)	19.5 ± 1.8	25.6 ± 2.8
Rim dips (number)	12.8 ± 1.4	12.0 ± 1.4

Data are expressed as mean ± S.E.M. s: seconds; m: meter

* $p < 0.05$ rat stress vs. controls.

measured as compared to novel cage exposure (baseline = 5.4ng/ml = 100%; t = 60 min: stress: 895.9 ± 68.9 vs. control: 403.9 ± 25.2% increase vs. baseline; Mann-Whitney, df=32; $p = 0.001$).

Body weight

All mice gained weight over the course of the experiment (± 3% increase on day 19; day 1 set to 100% bodyweight). Weight change in direct response to rat exposure or cage placement, was used as a marker for autonomous nervous system activation. Weight loss in stressed mice was significantly stronger (mean ± S.E.M. in mg; control: 43 ± 2 vs. stress: 59 ± 1; one way ANOVA, $F_{(1,31)}=39.894$, $p = 0.001$).

Additional experiment: Sucrose consumption and cage exposure change activity pattern

Mice altered their activity pattern during the dark active period in response to sucrose and exposure to their previous Macrolon Type II housing cage (main effect of group: % time - in open area: $F_{(2,33)}=10.099$, $p = 0.0001$; at feeder $F_{(2,33)}=3.624$, $p = 0.038$) with a different time course over days: interaction day*group (open area $F_{(6,99)}=3.935$, $p = 0.001$; at feeder $F_{(6,99)}=4.331$, $p = 0.001$; data not shown). Sucrose consumption was comparable to the main study (between 10 and 15ml/mouse). Only on day 5, when sucrose was present, time spent near the bottles increased by 50%; time spent at the feeder and in the open area decreased (all $p < 0.05$). Following two cage exposures during the light-on

hours on day 6, the time spent in shelter was increased during the next light-off period. Activity in the open area and at the feeder remained reduced on days 6, 7 and 8. Mice spent less time near the bottle and feeder, while more time was spent inside the shelter (all $p < 0.05$). Taken together, as long as sucrose was available, (foraging) activity was changed but returned to the level of undisturbed control mice thereafter. The removal of the mouse from the home cage PhenoTyper to the "previous housing cage", which we used as control procedure for rat stress in the main experiment, had long-lasting consequences, suppressing the activity pattern of the mice for the next two days.

Discussion

Longitudinal and continuous observation of behavioral activities of mice in their home cage resulted in two main findings: (1) During and after cessation of the rat stress paradigm exploration and foraging patterns changed; consumption of and preference for a sweet solution was less expressed than in control mice. Rat-stressed mice delayed the onset to explore the novel environment of the circular hole board and used perseverative exploration strategies. (2) The control procedure for rat stress, i.e. placing the mice for 1-2 hours in another cage, also resulted in a pronounced decrease and differential pattern of home cage activity. We conclude that experimental manipulations, which are generally considered as "minor", reveal themselves as long-lasting changes in the daily activity pattern of mice. In addition, repeated rat exposure leads to further distinct alterations of behavior in the familiar environment of the home cage, extending even to behavior in novel situations.

Effects of rat exposure on home cage behavior

Repeated, unpredictable and uncontrollable exposure of mice to rats can be considered as chronic psychological stress (Grootendorst et al. 2001a; Grootendorst et al. 2001b). Our results on reduced home cage activity in rat stressed mice are in line with findings from rat studies that reported reduced non-specific activity counts and running wheel activity in relation to stress procedures involving paired housing, food/water deprivation (Gorka et al. 1996), repeated social conflict (Meerlo et al. 1999) and inescapable electric shocks (Desan et al. 1988; Stewart et al. 1990). However, the current set-up allowed specification of the behavioral changes and their development over time.

Stressed mice spent more time in the shelter, spent less time and traveled less distance in the open area of the home cage than mice of the control group. Principal Component Analysis (PCA) revealed that exploration of the open area (Factor 1) is independent from activity related to foraging (Factor 2), i.e. time at the feeder and drinking bottles. Stressed mice showed a decrease in both exploratory and foraging activity. We interpreted these findings in the following way: once a stressed mouse leaves the shelter, its focus is on obtaining food and water, a primary life necessity, while suppressing exploratory activity that is not related to foraging. We conclude that stressed mice kept their actual food and water intake at the same level as control mice, but had adapted a faster collection of the consumables. Food might also have been transported to the shelter. While directly after the rat exposure mice had lost more weight than controls, the body weight remained comparable. This is another indication that stressed mice consumed as much or even more than controls. It might be the case that the food is metabolized differently, resulting in a differential distribution of fat and muscle tissue (Moles et al. 2006).

The effects of stress revealed by the PCA are supported by theories on the economy of behavior. These predict that animal's trade-off their foraging effort in relation to variation of predation risk ("predation risk allocation hypothesis"; (Lima and Bednekoff 1999). Rats are not actual predators of mice, but in nature, mice and rats avoid to share the same living environment. Animals need to forage for food to meet their energy demands, while at the same time they need to minimize the risk of being exposed to a life-threatening situation, i.e. the predator or the threat of a possible predator. We may argue that reduction of exploratory activity has an adaptive value by temporarily decreasing the risk of predation (Norrdahl and Korpimaki 1998).

In contrast to nature, consumables are available *ad lib* in the experimental setting. Moreover, the actual exposure to rats took place in a distinctly different environment. The change in nocturnal activity patterns reveals the long-lasting consequences of the "rat experience" and leaves the impression that also the familiar "safe" environment of the home cage became threatening.

Observing the development of exploration and foraging over the course of rat exposures, we found that the initially lower exploratory activity returned to baseline levels after the tenth rat exposure. However, Factor 2 (foraging activities) remained lower, indicating that the effects of rat stress have distinct effects on the time pattern of behaviors.

Effect of rat stress on sucrose consumption and preference

Measurement of sucrose intake or preference is currently in widespread use in preclinical psychopharmacology for predicting sensitivity to rewards. Animal models of chronic stress in rats generally report a decrease in sucrose consumption as a measure for anhedonia (Dalm et al. 2000; Pothion et al. 2004; Strekalova et al. 2004; Anisman and Matheson 2005; Willner 2005). However, preference for the sweet solution seems to be a more appropriate marker for anhedonia. As expected, mice showed an impressive consumption of (12ml) and preference for (85%) the 5% sucrose solution. Control mice further increased their consumption (up to 144%) and preference over the course of the experiment. Stressed mice decreased the sucrose consumption in the initial phase of rat stress and baseline levels were reached one day after the cessation of rat exposures. Mice housed for 3 weeks with rats did not increase their sucrose consumption either (Calvo-Torrent et al. 1999). Although all mice preferred sucrose over water, the preference for sucrose increased in the course of the experiment only for control but not rat stressed mice. There is a clear time-dependent pattern in the development of increasing consumption of and preference for sucrose in the control mice which is absent in the rat stressed mice. During the stress paradigm (on days 6 and 10) stressed mice spent less time near the bottles than controls, which most likely reduced their fluid intake. However, since the preference of the stressed mice for sucrose did not change in the course of the experiment, and water intake was comparable to controls we feel confident that rat stress affected the hedonic properties of sucrose. Stressed mice did not increase their preference and consumption like controls. Consequently, we assume that the rat stress procedure affected the reward system, also somewhat counteracting the addictive properties of sucrose (Avena et al. 2008).

The volume overload of 300% due to sucrose drinking most likely affected the body's fluid and energy balance. Sucrose by itself is rich in energy, which is utilized directly, stored in adipose tissue or secreted from the body (Peters et al. 2004). Drinking sucrose might have lowered stress-induced corticosterone secretion as shown by Bell and colleagues (Bell et al. 2000). Indeed, absolute corticosterone values in response to rat exposure were lower than measured in previous studies (Grootendorst et al. 2001a; Grootendorst et al. 2001b). Our additional experiment revealed, that the rat stress control procedure (placement in another cage) reduced the nocturnal activity for at least two days, while sucrose overload affected the activity pattern only on the day of consumption. Therefore, the reduced consumption and lesser preference for sucrose is a distinct feature of the rat stressed mice.

Exploration of a novel environment

Rat stressed and control mice showed similar locomotor activity (distance walked and velocity) on the circular hole board, while home cage activity patterns were lower in the stressed mice. Locomotor activity in response to a novel environment cannot be directly compared to activity in a familiar surrounding. In both conditions, a different type of activity is measured, next to the difference in duration and time of measurement. Instead in locomotor activity, the effect of stress was observed at a different level. Interestingly, the exploration pattern of mice on the circular hole board was changed. Stressed mice alternated more often between serial (sequential hole visits) and perseverative (repetitive visits of the same hole) search strategies. While control mice readily set out to explore, stressed mice were slower in starting to visit holes and performing rim dips, indicative for more anxiety-related behavior. Exploration of the holes and the border are important to locate possible routes of escape from the open, unprotected environment. We may assume that the exploration pattern of stressed mice decreases the possibility to locate an escape route. This might also relate to the impairment of cognitive abilities that have been observed in previous studies (Grootendorst et al. 2001b).

Effects on home cage activity of the control mice

The activity of mice *before* the intervention resembled the nocturnal pattern described in previous studies (de Visser et al. 2005; de Visser et al. 2006). Unexpectedly, the control procedure for rat stress, i.e. exposure to a 'familiar' cage for one to two hours reduced activity patterns, albeit to a lesser degree than in the rat stressed group. Others have shown in rats that even routine control procedures like placement into a clean cage, can induce stress system activation and affect behavior (Meerlo et al. 1996; Duke et al. 2001; Balcombe et al. 2004). Placing a rat in a novel cage for 1h in the same room, at the same time when another rat was defeated had a dramatic effect on the body temperature during the day and the activity during the night (Meerlo et al. 1996). Importantly, the results of our additional experiment support the long lasting reduced nocturnal activity of the mice. Exposing the mice to the circular hole board for 5 minutes during the light period of day 19, resulted again in a reduction of activity thereafter. Exploratory and foraging factors returned to the level of day 6, when the mice were exposed for two hours to a novel cage.

Like in our study, most laboratories conduct experiments during the light, inactive period of rodents. Others have shown that the effect of stressors on stress system activation depend on the kind of stress and time of day when applied (Akana et al.

1986; Retana-Marquez et al. 2003). The effects of experimental "control" manipulations and even a short-lasting behavioral test go unnoticed in most studies that compare treatment effects between groups. The longitudinal design of the present study revealed the strong impact of common handling procedures.

Conclusions

The home cage PhenoTyper design includes the measurement of baseline behavior and thus, allowed in a longitudinal setting *within* and *between* group comparisons. We found evidence that already "basic" experimental manipulations like relocation of the mice to another cage, performing a short-lasting behavioral exploration task, have strong and long-lasting influences on the organization of circadian behavioral activity. We now know that the rat stress effect is a combination of rat exposure and accompanying experimental conditions like transport, handling, disturbance of the light phase resting and sleep behavior. Rat exposure resulted in (1) a stronger and differential inhibition of exploration and foraging activity in the home cage than the control procedure, (2) a decreased response to reward expressed by sucrose drinking and sucrose preference and (3) a specific exploration strategy in a novel environment. Behavioral and neuroendocrine changes might still be in the range of adaptive responses to stress. We conclude that our rat exposure design shows the potential for a mouse model of chronic stress and will allow to study and elucidate mechanisms underlying the inhibition of behavior in relation to stress system activation.

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