ELSEVIER

Journal of Neuroscience Methods 143 (2005) 169–177

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

The grooming analysis algorithm discriminates between different levels of anxiety in rats: potential utility for neurobehavioural stress research

Allan V. Kalueff*, Pentti Tuohimaa

Department of Anatomy of the Medical School, Department of Clinical Chemistry of the University Hospital, University of Tampere, Tampere 33014, Finland

Received 15 August 2004; received in revised form 23 September 2004; accepted 4 October 2004

Abstract

Stress has long been known to affect grooming in rodent species, altering both its activity measures and behavioural microstructure. Since stress disturbs a general pattern of self-grooming uninterrupted cephalocaudal progression, the grooming analysis algorithm (Kalueff and Tuohimaa, Brain Res. Protocols, 2004; 15: 151–8) was previously designed for mice to enable the detection of stress by measuring alterations in grooming microstructure in different test situations. Since mice and rats are known to differ in their behaviours, including grooming, the aim of the current study was to test our approach in rats and evaluate the utility of this method for differentiation between high- and low-stress situations. For this, we have developed the rat grooming analysis algorithm (based on ethological analysis of incorrect transitions contrary to the cephalocaudal rule, interrupted grooming activity and the assessment of the regional distribution of grooming) and applied this algorithm to the light-exposed (high stress) and dark-exposed (low stress) groups of rats. Here, we show that the percentage of 'incorrect' transitions between different grooming patterns, the percentage of interrupted grooming bouts and altered regional distribution of grooming (less caudal grooming, more rostral grooming) may be used as behavioural markers of stress in rats. Our results suggest that this method can be a useful tool in neurobehavioural stress research including modelling stress-evoked states, psychopharmacological or behavioural neurogenetics research in rats.

Keywords: Grooming; Rats; Behavioural microstructure; Ethological analysis; Stress

1. Introduction

Self-grooming is an ancient innate body care behaviour that is represented across most animal species (Fentress, 1977; Greer and Capecci, 2003; Spruijt et al., 1992). Grooming is a frequently performed behaviour by small felids, bovids, cervids and primates (Eckstein and Hart, 2000; Hart and Pryor, 2004), and is an important part of rodent behavioural repertoire (Berridge and Whishaw, 1992; Berridge et al., 1987; Van Erp et al., 1995). In rodents, grooming is a complex, ethologically rich ritual, which normally proceeds in a cephalocaudal direction and consists of several stages, including licking the paws, washing movements over the head, fur licking, and tail/genitals cleaning (Berridge and Aldridge, 2000a,b; Eguibar and Moyaho, 1997; Fentress, 1977). Many neuromediators and hormones as well as multiple regions in the brain appear to be involved in the regulation of grooming behaviours (Bolivar et al., 1996; Bressers et al., 1998; Cromwell and Berridge, 1996; Cromwell et al., 1998; Van Erp et al., 1995). Grooming is highly sensitive to various stressors, psychotropic drugs and genetic manipulations (Dunn et al., 1987; Gerlai et al., 1998; Choleris et al., 2001; Spruijt et al., 1992), and has long been studied in laboratory rodents, including mice and rats (Crawley et al., 1997; Jarbe et al., 2002; Kalueff et al., 2004a,b; Van de Weerd et al., 2001; Van Erp et al., 1995). In rodents, grooming plays an important role in behavioural adaptation to stress, including stress-coping

* Corresponding author. Tel.: +358 3 2156640; fax: +358 3 2156170.
E-mail address: avkalueff@inbox.ru (A.V. Kalueff).

0165-0270/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2004.10.001

and de-arousal (Eguibar et al., 2003; Kametani, 1988; Sachs, 1988; Spruijt et al., 1992).

Overall, rodents' grooming can be dissected at a number of levels, ranging from individual limb segment kinematics to the sequencing of grooming patterns and their association with other (non-grooming) behaviours (Bolivar et al., 1996; Komorowska and Pisula, 2003; Moyaho et al., 1995). Although many studies have analysed different aspects of grooming and its behavioural organization (Van Erp et al., 1994, 1995; Bressers et al., 1998; Cromwell and Berridge, 1996; Cromwell et al., 1998), the exact role of grooming behaviour in stress is still not well understood (Homberg et al., 2002; Komorowska and Pellis, 2004). Indeed, it has long been known that rodents' grooming activity can be generally increased in two opposite situations: in high- and low-stress (Kalueff, 2002; Katz and Roth, 1979). 'Comfort' grooming is a ritual occurring as a transition from rest to activity, and under spontaneous or low stress conditions following a cephalocaudal rule (Fentress, 1977; Kalueff, 2000; Spruijt et al., 1992). However, mild stress such as exposure to novel environment has also been known to induce grooming in rats (Eguibar and Moyaho, 1997; Escorihuela et al., 1999; Moody et al., 1988; Van Erp et al., 1994). Notably, this stress-evoked 'displacement' grooming is ethologically different from no-stress grooming, and characterised by frequent and rapid short bursts (Fentress, 1977; Kalueff, 2000; Moyaho and Valencia, 2002). As such, it is becoming necessary to assess in more detail stress-evoked grooming behaviours and their behavioural microstructure in rodent species commonly used in neurobehavioural research, such as mice and rats.

Although grooming in rodents plays a very important role in various behavioural models of stress (Van Erp et al., 1994, 1995), in many studies this behaviour has merited little scrutiny, frequently addressed only cursorily among other measures (e.g., Aguilar et al., 2003, Hossain et al., 2004; Jarbe et al., 2002; Ohl et al., 2001; Van de Weerd et al., 2001). In most studies, general characteristics of grooming behaviour (latency to onset, frequency and duration) have been described (Barros et al., 1994; Espejo, 1997; Moody et al., 1988); however, few reports analyse the organization (patterning) of grooming in different stressful situations (Kametani, 1988; Komorowska and Pisula, 2003). Since rodents' self-grooming is increased by both stress and comfort conditions (File et al., 1988; Lawler and Cohen, 1988; Moody et al., 1988), the traditional 'quantitative' measures of grooming may be insufficient for correct data interpretation and analysis (Kalueff, 2000, 2002). Indeed, although stress elicits grooming activity in rodents, there are many data showing that reduced levels of stress may also lead to this phenomenon. For example, 'anxiolytic' sudden darkness (Nasello et al., 2003) in rats is seen together with increased time spent grooming. Some anxiolytics (ethanol, muscimol) have been reported to increase, while anxiogenics (bicuculline) to inhibit grooming in rats (File et al., 1988; Osborne et al., 1993; Perier et al., 2002). In mice, anxiolytic chlordiazepoxide and diazepam activated grooming in the elevated plus maze (Rodgers et al., 2002) and in the central area of the open field (Choleris et al., 2001), respectively. Furthermore, our recent studies also found no clear correlation between grooming scores and anxiety in mice. For example, lower grooming activity was seen in more anxious 129S1 versus non-anxious C57BL/6 mice (Kalueff and Tuohimaa, 2004b, also see similar results in Hossain et al., 2004). C57BL/6 mice demonstrated similar degree of grooming activation in both low-stress (novel box) and high-stress (social encounter with an unfamiliar male) tests (Kalueff and Tuohimaa, 2004a). Finally, more stressful situation (exposure to the elevated plus maze) produced less grooming in these mice than did a relatively weak stressor (exposure to a familiar box) (Kalueff and Tuohimaa, 2004a). Taken together, these data indicate that grooming 'quantitative' measures in rodents may not always reliably reflect the level of stress, and that additional grooming measures (such as its 'qualitative', or patterning characteristics) are also necessary in order to assess animals' stress-evoked behaviours.

The patterned structure of rodent grooming makes it particularly attractive for neurobehavioural stress research (Komorowska and Pellis, 2004). It has been recently suggested that grooming behavioural microstructure undergoes predictable changes in stressful situations (Kalueff, 2000; Komorowska and Pellis, 2004), thus making it possible to ethologically dissect different types of rodents' grooming activity. For this, we have previously designed the mouse grooming analysis algorithm, based on the ethological differences between comfort- and stress-evoked grooming (Kalueff and Tuohimaa, 2004a). This 'qualitative' approach used differential registration and analysis of grooming behavioural microstructure, including (1) transitions between self-grooming patterns and (2) interruptions of grooming bouts in mice exposed to different stressors. In a striking contrast to grooming 'activity' measures, this algorithm worked consistently in all experimental models, demonstrating clear impairments of grooming patterning in anxious animals (129S1 versus C57BL/6 mice, stressed versus non-stressed C57BL/6 mice) and showing no such alterations in low- or no-stress situations (Kalueff and Tuohimaa, 2004a,b).

Importantly, although mice and rats share many common behavioural features, including grooming (Whishaw et al., 2001), it was unclear whether this approach may be used for stress-oriented research in rats. Indeed, some differences have been reported for various behaviours in these two species, including grooming. For example, rats generally display complex and organized grooming patterns, with more symmetrical movements and transitions between stages (Berridge, 1990). Together, this suggests that grooming microstructure in mice may be much simpler than that in rats, see Whishaw et al. (2001) for details. Given these findings, it was possible to assume that stress sensitivity of grooming microstructure in rats may markedly differ from that previously reported in mice (Kalueff and Tuohimaa, 2004a).

In the present study, we wanted to examine whether shifts in grooming behavioural microstructure (detected by our

grooming analysis algorithm) can be used to assess different levels of stress in rats. To induce different (high and low) levels of stress, we used the light–dark paradigm (Crawley, 1999), validated for rats and widely used in behavioural neuroscience (Salome et al., 2002). This two-chambered test consists of two different compartments – highly aversive 'light' compartment (brightly lit box with transparent walls) and more protective 'dark' compartment (black box). In our study, we first randomly exposed rats to the light or dark box (to induce high- or low-stress, respectively), and then assessed their stress-evoked grooming in the actimeter test. Here we show that behavioural microstructure of rat grooming is indeed very sensitive to the level of stress, and that stress-induced alterations in rat grooming activity are in many ways similar to those previously reported in mice (Kalueff and Tuohimaa, 2004a). Overall, our study shows that this method allows detection of stress by measuring alterations in grooming patterns (microstructure), and can be a useful tool for neurobehavioural stress research in rats.

2. Materials and methods

2.1. Subjects

Twenty-two male Wistar rats (2 months old, 220–240 g, University of Tampere, Finland) were maintained in a virus/parasite-free facility under conditions of controlled temperature ($22 \pm 2\,^{\circ}C$), humidity (60%) and a 12:12 h light–dark cycle (lights on at 7:00 h). The animals were housed in pairs in standard laboratory cages, with water and food available ad libitum.

2.2. Apparatus and procedure

All testing was conducted between 17:00 and 19:00 h. On the day of the experiments, animals were transported to the dimly lit laboratory and left undisturbed for 2 h prior to exposure to the light–dark boxes. The light–dark test was a Plexiglas box consisting of two compartments – the transparent (light) and the black (dark) boxes (30 cm × 30 cm × 30 cm each). The light box was brightly lit (75 W lamp, 10 cm above the top) and connected to the dark box by a sliding door (this door was not used in the present study). Each rat was placed separately in the light (high-stress group, $n = 11$) or dark (low-stress group, $n = 11$) box for 5 min. Immediately after this exposure, each rat was placed separately in the actimeter test, where its behaviour was recorded for 5 min. The actimeter test was a glass cylinder (20 cm in diameter; 40 cm in length). During the testing sessions, the experimenter remained standing in front of (and 2 m away from) the testing boxes. Between subjects, each apparatus was thoroughly cleaned (wet and dry cloths); to remove olfactory stimuli, each apparatus was cleaned with a 10% ethanol solution and dried with paper towelling. All animal experiments were performed in full compliance with the Finnish laws on animal experimentation and approved by the Ethical Committee of the University of Tampere.

2.3. Behavioural analysis

2.3.1. Non-grooming behaviours

Defecation and urination index (DU, the sum of two visceral behaviours: the number of defecation boli deposited and urination spots) was scored as the conventional emotionality index in all animal groups in the light–dark and the actimeter tests. In addition to measuring grooming and visceral behaviours in the actimeter test, we also assessed general vertical exploratory activity (VR, the number of vertical rears) as the conventional stress-sensitive behavioural measure.

2.3.2. Grooming ethological analysis

Three gross measures of grooming activity were evaluated in this study: (i) the latency (s) to start grooming, LG, (ii) the number of grooming bouts, NB (the number/frequency of grooming episodes), and (iii) total time (s) spent grooming, TS. A bout consisted of a single pattern (single-area bout, SAB) or was composed of several patterns (multiple-area bout, MAB): (1) paw licking; (2) nose/face grooming (strokes along the snout); (3) head washing (semicircular movements over the top of the head and behind the ears); (4) body grooming/scratching (body fur licking and scratching the body with the hind paws); (5) leg licking and tail/genitals grooming (licking of the genital area and tail), according to Kalueff and Tuohimaa (2004a); 0 indicated no grooming. The number of grooming patterns (NP) and the number of interruptions (NI) were calculated for all groups (interruptions longer than 5 s determined separate, independent grooming bouts). A grooming bout was considered 'interrupted' (IB), if at least one interruption was recorded within its transitions. The number (NIB) and the percentages of interrupted bouts (% IB = NIB/NB) were assessed in this study. A 'complete' bout (CB) consisted of the following sequence of patterns: 0–1–2–3–4–5–6–0; all other bouts were considered 'incomplete' (ICB). The number (NICB) and the percentages of incomplete bouts (% ICB = NICB/NB) were assessed in this study.

Transitions between grooming patterns were assessed using the transition matrix as described earlier (Kalueff and Tuohimaa, 2004a). 'Correct' transitions (CT) adhered to the cephalocaudal progression: (0–1), (1–2), (2–3), (3–4), (4–5), (5–6), and (6–0); 'incorrect' transitions (IT) included all other possible transitions between grooming patterns. Total number of transitions (T) was the sum of correct and incorrect transitions (T = CT + IT). Four main types of IT were analysed in this study: (i) aborted, IT(a) (prematurely terminated, e.g. 3–0, 4–0); (ii) skipped, IT(s) (e.g. 1–6, 2–5); (iii) reversed, IT(r) (e.g. 3–2, 4–1, 5–2), and (iv) incorrectly initiated, IT(i) (e.g. 0–4, 0–5). The percentages of incorrect transitions (% IT) were calculated for both groups of rats (% IT = IT/T). The numbers of incorrect transitions (total and for each category) were assessed in this study, and the percentages of incorrect

transitions of each type, i.e. reversed IT(*r*), skipped IT(*s*), aborted IT(*a*) and incorrectly initiated IT(*i*) of total transitions (*T*) were calculated in this study (% IT(. . .) = IT(. . .)/*T*).

The grooming analysis algorithm also included a set of ethological indices derived from the following calculations: average durations of a single bout (ADB) and pattern (ADP) were calculated as total time spent grooming divided by the number of bouts (ADB = TS/NB) and patterns (ADP = TS/NP), respectively. Average number of transitions per bout (ATB) and pattern (ATP) were calculated as the number of transitions divided by the number of bouts (ATB = T/NB) and patterns (ATP = T/NP), respectively. Average number of interruptions per bout (AIB) was calculated as the number of interruptions divided by the number of bouts (AIB = NI/NB).

2.3.3. Regional distribution of grooming

In order to assess the regional distribution of grooming in rats, we separately analysed their grooming activity directed to the following five anatomic areas: forepaws, head, body, hindlegs and tail/genitals (caudal grooming). Rostral grooming included forepaw (preliminary rostral grooming, according to Komorowska and Pellis (2004)) and head grooming. The percentages of (i) total grooming patterns, (ii) time spent grooming, and (iii) interruptions were calculated for each anatomic area. In addition, each grooming bout was categorised as being directed to (i) multiple regions or (ii) a single region, according to Eckstein and Hart (2000), and the percentage of grooming bouts and the percentage of time spent grooming were calculated for both categories.

2.3.4. Statistical analysis

All data are presented as mean ± S.E.M. Data were analysed by Mann–Whitney two-tailed *U*-test. In all tests, a probability of less than 0.05 was considered statistically significant.

3. Results

In the light box, the rats demonstrated significantly more defecations and urinations, compared to the dark-exposed animals (Table 1). In the actimeter test, the light-exposed group demonstrated significantly less vertical rears and more defecation and urination scores (although this did not reach significance, $P = 0.07$).

Table 2 shows grooming activity of rats after their exposure to the light or dark boxes of the light–dark test. Following exposure to a high stress evoked by the light box (novelty + open lit area), the traditional gross measures of grooming (the number and the duration of grooming bouts) in the actimeter test increased significantly compared to the group exposed to a low stress test (dark box). As can be seen in Table 1, the latency to start grooming was predictably shorter in more anxious light-exposed rats, while the average duration of a single bout was similar in both groups.

Analysis of grooming patterns using our grooming analysis algorithm shows that anxious light-exposed rats display significantly more patterns and more interruptions of grooming activity, compared to the low stress group (Table 2). However, average duration of a single pattern was unaltered in both groups.

Analysis of transitions between grooming patterns again shows that following exposure to the light box, the number of transitions increased significantly in these rats (Table 2) compared to their less stressed (dark-exposed) counterparts. While average number of transitions per pattern was only slightly higher in the first group, we found a marked increase in the percentage of incorrect transitions in more stressed group. The use of grooming analysis algorithm also revealed the increase in reversed and aborted incorrect transitions in these rats, compared to their less stressed counterparts.

As can be seen in Table 2, a detailed analysis of grooming bouts in both groups shows that the light-exposed rats generally display more interrupted bouts and a clear tendency to more incomplete bouts. Average numbers of patterns and transitions per a single bout were similar in both groups, also showing a tendency to more interruptions per bout.

Moreover, our experiments also show that the regional distribution of grooming behaviours was markedly affected by stress, manifest in more forepaw grooming patterns and less tail and genitals grooming in the high-stress group (Table 3). Consistent with this, these stressed rats spent significantly more time grooming forepaws and less time grooming tail and genitals, compared to their less anxious dark-exposed counterparts. Furthermore, analysis of the regional distribution of interruptions clearly shows the rostral character of grooming interruptions in more stressed rats. Finally, both rat groups displayed essentially the same percentages of single- and multiple-area bouts and time spent grooming (Table 3).

4. Discussion

The results of this study show that, in line with traditional interpretation of the light–dark paradigm (Crawley, 1999; Crawley et al., 1997), this procedure induced two different levels of stress, as assessed by the number of urination spots and defecation boli deposited (Table 1). This confirms that the rats subjected to the light box in this study were more anxious than their counterparts subjected to the dark box. Consistent with this, the rats from the first group showed fewer vertical rears in the actimeter test – the behavioural response traditionally considered as a reliable marker of stress in rodents (Belzung, 1999; Crawley et al., 1997; Espejo, 1997). Furthermore, this group demonstrated shorter latencies to start grooming, and a dramatic increase in grooming frequency and duration (Table 2). These alterations in rats' grooming have long been considered as behavioural markers of stress (Kalueff, 2000; Moyaho and Valencia, 2002). Taken together, this allowed us to conclude

Table 1
Non-grooming behaviours in male Wistar rats exposed to the light/dark box and the actimeter test ($n = 11$ in each groups)

Behavioural measures	Light-induced stress group	Dark-induced stress group	*P*
Light/dark exposure test			
Defecation and urination index, DU	0.8 ± 0.2	0.1 ± 0.1	0.009
Actimeter test (following the light/dark exposure)			
Defecation and urination index, DU	0.5 ± 0.3	0.1 ± 0.1	0.07 (NS)
Number of vertical rears, VR	14 ± 4	26 ± 5	0.01

Data are expressed as mean ± S.E.M. *P*-difference between the groups (*U*-test), DU index in the sum of defecation boli deposited and urination spots.

that the two groups of rats used in the present study exhibited different levels of the induced stress.

We then applied our grooming analysis algorithm in order to assess in detail the behavioural microstructure of the rat stress-evoked grooming. In general, the idea of analysing the rat grooming in the actimeter test after the exposure to the light–dark apparatus was useful for two main reasons. Firstly, as already mentioned, the light–dark test is a convenient standard paradigm, in which two different levels of stress can easily be produced and assessed. Secondly, more specific analysis of stress-induced grooming behaviours in the subsequent relatively simple actimeter test enables higher grooming scores and minimizes possible confounding effects of other (non-grooming) behaviours. As expected, the grooming analysis algorithm revealed a marked shift in grooming behavioural patterns, significantly increasing the percentage of incorrect transitions and interrupted bouts in more anxious rats. Finally, our present data show that these stress-evoked alterations in grooming microstructure in rats parallel changes in their gross grooming measures, thus further supporting the validity of our results. Together, this shows that the present method may be particularly effective in detection of stress-evoked abnormal grooming patterns in rats tested in different experimental models of stress.

Does the behavioural organization of stress-evoked grooming activity differ in mice and rats? Comparing our results with previously published own grooming data in mice (Kalueff and Tuohimaa, 2004a,b; Kalueff et al., 2004a), we note that stressed rats and mice spent similar amount of test time engaged in self-grooming (~8–14%). Likewise, mice and rats exposed to low stress situations, also demonstrated similar (although much lower) levels of grooming activity.

Table 2
Behavioural alterations in grooming activity in the actimeter test in the light- and the dark-exposed Wistar rats ($n = 11$ in each group)

Grooming measures	Light-induced stress group	Dark-induced stress group	*P*
Traditional gross measures			
Latency to start grooming (s), LG	201 ± 50	360 ± 58	0.01
Total number of bouts, NB	4.8 ± 0.8	2.4 ± 0.5	0.02
Total time spent grooming (s), TS	53 ± 8	24 ± 5	0.001
Average duration of a single bout (s), ADB = TS/NB	11 ± 2	10 ± 2	0.6 (NS)
Patterns			
Total number of patterns, NP	14 ± 2	8 ± 1	0.02
Number of interruptions of grooming, NI	1.6 ± 0.5	0.2 ± 0.1	0.01
Average duration of a single pattern (s), ADP = TS/NP	3.8 ± 0.6	3 ± 0.7	0.3 (NS)
Transitions between patterns			
Total number of transitions, T	18 ± 3	9 ± 1	0.02
Average transitions per pattern, ATP = T/NP	1.3 ± 0.4	1.1 ± 0.1	0.6 (NS)
Percent of incorrect transitions (IT), % IT (of total transitions, *T*)			
Reversed IT, % IT(*r*)	13 ± 2	2 ± 1	0.01
Skipped IT, % IT(*s*)	6 ± 1	6 ± 1	0.9 (NS)
Aborted IT, % IT(*a*)	20 ± 3	13 ± 1	0.03
Incorrectly initiated, % IT(*i*)	8 ± 1	7 ± 1	0.2 (NS)
% Total, % IT = IT/T = % IT($r + s + a + i$)	47 ± 5 (100)	28 ± 3 (100)	0.04
Bouts			
Number of interrupted bouts, NIB	1.2 ± 0.4	0.2 ± 0.1	0.03
% Interrupted bouts, % IB = NIB/NB	25 ± 8	8 ± 4.8	0.04
Number of incomplete bouts, NICB	4.5 ± 0.4	1.8 ± 0.2	0.001
% Incomplete bouts, % ICB = NICB/NB	94 ± 5	75 ± 9	0.08 (NS)
Average number of patterns per bout, APB = NP/NB	2.9 ± 0.4	3.3 ± 0.4	0.4 (NS)
Average number of transitions per bout, ATB = T/NB	3.8 ± 0.6	3.8 ± 0.4	0.9 (NS)
Average interruptions per bout, AIB = NI/NB	0.4 ± 0.1	0.1 ± 0	0.07 (NS)

Data are expressed as mean ± S.E.M. *P*-difference between the groups (*U*-test).

Table 3
Regional distribution of grooming in the actimeter test in the light- and the dark-exposed Wistar rats ($n = 11$ in each group)

Regional distribution	Light-induced stress group	Dark-induced stress group	*P*
Grooming patterns (% of total number of patterns, NP)			
Forepaws	38 ± 5	26 ± 4	0.4 (NS)
Face/head	31 ± 8	31 ± 7	0.6 (NS)
Rostral grooming (paws + face/head)	69 ± 7	57 ± 5	0.1 (NS)
Body	18 ± 4	17 ± 3	0.5 (NS)
Hindlegs patterns	7 ± 1	10 ± 1	0.3 (NS)
Tail and genitals (caudal grooming)	7 ± 1	16 ± 2	0.02
Grooming duration (% of total time spent grooming, TS)			
Forepaws	45 ± 6	33 ± 5	0.03
Face/head	26 ± 4	24 ± 3	0.3 (NS)
Rostral grooming (paws + face/head)	71 ± 5	57 ± 4	0.04
Body	15 ± 4	17 ± 4	0.5 (NS)
Hindlegs	6 ± 1	6 ± 1	0.6 (NS)
Tail and genitals (caudal grooming)	8 ± 1.5	20 ± 3	0.02
Interruptions of grooming (% of total number of interruptions, NI)			
Forepaws	50 ± 11	33 ± 3	0.04
Face/head	32 ± 5	0	0.01
Rostral grooming (paws + face/head)	82 ± 7	33 ± 3	0.001
Body	6 ± 1.5	0	0.03
Hindlegs	6 ± 2	33 ± 3	0.06 (NS)
Tail and genitals (caudal grooming)	6 ± 2	0	0.01
Regional characteristics of grooming bouts, % of total bouts (NB) or time spent (TS)			
Single-area bouts, % SAB	34 ± 5	34 ± 7	0.8 (NS)
Single-area duration, % SAD	66 ± 9	66 ± 7	0.8 (NS)
Multiple-area bouts, % MAB	17 ± 4	16 ± 5	0.8 (NS)
Multiple-area duration, % MAD	83 ± 7	84 ± 11	0.7 (NS)

Data are expressed as mean ± S.E.M. *P*-difference between the groups (*U*-test). % SAB = number of single-area bouts/NB; % SAD = single-area bouts duration/TS; % MAB = number of multiple-area bouts/NB; % MAD = multiple-area bouts duration/TS.

However, in line with the previously published observations (Berridge, 1990), we also found that grooming behaviour in rats appeared to be more complex and better organized, showing fewer but longer bouts, and lower percentages of incomplete and interrupted bouts. In addition, essentially the same increase in incomplete and interrupted bouts occurred in stressed mice and rats, compared to their non-stressed controls (Kalueff and Tuohimaa, 2004a) (Table 2). Collectively, these observations not only confirm common behavioural organization of stress-evoked grooming in these two species, but also prove the potential utility of ethological analysis of rodent grooming microstructure for the detection of stress-induced behavioural alterations in mice and rats.

At first glance, the dark box exposure used in this study is similar to the sudden darkness test (Nasello et al., 2003) known to reduce anxiety and activate grooming in rats. Clearly, this phenomenon supports the notion that increased grooming activity does not always reflect higher levels of stress (Kalueff, 2000; Kalueff and Tuohimaa, 2004a). However, it is possible that the dark exposure used here differs from the sudden darkness procedure, especially since we did not find increased grooming activity in the dark-exposed rats (Table 2). However, we did find a significantly higher level of tail/genital grooming – the effect similar to that induced by the sudden darkness (Nasello et al., 2003). Although these interesting phenomena need further investigation, they are generally consistent with the idea of low stress in the dark-exposed rats (Nasello et al., 2003) (Tables 1–3). Moreover, this 'anxiolytic' interpretation of the increased caudal grooming in both studies is in line with predominantly 'rostral' nature of rodent grooming evoked by stress (Van Erp et al., 1995; Komorowska and Pisula, 2003; Kalueff and Tuohimaa, 2004a). Consistent with this, we found that rostral grooming (forepaws, face and head) was significantly higher in more anxious light-exposed group (∼70%) compared to the dark-exposed rats (∼57%), Table 3.

Furthermore, since interruptions in grooming activity are considered as behavioural markers of stress in rodents (Kalueff, 2000; Kalueff and Tuohimaa, 2004a), the fact that the light-exposed rats displayed eight-fold more interrupted grooming bouts, especially while grooming rostrally (Tables 2 and 3), further confirms their higher anxiety profile compared to the dark-exposed rats. Moreover, since high anxiety in rats has long been associated with frequent prematurely terminated (aborted) grooming bouts (Komorowska and Pisula, 2003), the increased percentage of incorrect aborted transitions in the light-exposed group (Table 2) clearly supports the idea that grooming behavioural microstructure in rats is very sensitive to the level of stress. In line with this conclusion, we also found a dramatic increase in the number of reversed transitions (another behavioural marked of stress-evoked grooming; (Kalueff, 2002)) in the light-exposed group. Finally, all these alterations in grooming microstructure appear to parallel non-grooming stress-

related behaviours (Table 1), further strengthening the validity of our approach. Taken together, the results of the present study in rats confirm that alterations in rodents' grooming microstructure can be used as reliable behavioural markers of stress.

In summary, given the similarity of mouse and rat grooming behaviours (Berridge, 1990; Berridge and Aldridge, 2000a,b), our findings further support the idea of common principles of behavioural organization of stress-evoked grooming in small laboratory rodents, such as mice and rats. Importantly, the data presented in Tables 2 and 3 show that in high- and low-stress situations our algorithm in rats was able to detect the level of stress, as assessed by increased percentages of (i) incorrect transitions between different patterns; (ii) duration of incorrect patterns; (iii) interrupted bouts; and (iv) altered regional distribution of grooming (more rostral, less caudal grooming). Importantly, these findings are consistent with several recent studies (Komorowska and Pellis, 2004; Komorowska and Pisula, 2003) which, using different ethological approaches, also demonstrated the disruption of the cephalocaudal grooming progression in rats subjected to novelty stress. In general, the results presented here show that a detailed grooming analysis based on the algorithm previously designed for mice (Kalueff and Tuohimaa, 2004a) may be a valuable tool in neurobehavioural stress research in rats.

What can be the potential practical applications of this method? First, it is possible to speculate that this method may not only detect stress-evoked alterations in rat grooming microstructure, but also measure the degree of stress evoked by different tests. Clearly, the idea of grooming-based 'stress-meter' in rodents needs further investigation to be confirmed, but, if true, may represent an important practical application of our algorithm. To some extent, the results of the present study and our recent data on mice (Kalueff and Tuohimaa, 2004a,b; Kalueff et al., 2004b), showing consistent stress-dependent shifts in grooming microstructure, indirectly support this contention. Second, given similar behavioural organisation of grooming in mice and rats, and also our recent findings in mutant mice (Kalueff and Tuohimaa, 2004a), we can suggest that this method may be able to detect stress-evoked alterations in grooming in different rat strains used in behavioural neuroscience research. Although not directly studied here, this also implies the potential utility of our method for behavioural phenotyping of various mutant rats, especially those displaying abnormal emotional behaviours and/or grooming (e.g. Kobayashi et al., 1996). Third, since various physiological (Spruijt et al., 1992) and genetic manipulations (Kobayashi et al., 1996) in rats may impair their behavioural organization, including grooming, the sequential analysis of grooming according to our protocol is also likely to detect various motor/coordination anomalies in such animals.

Another potential application of our method may be ethological analysis of age-related behavioural alterations in rats. Our general knowledge of the age-related progression of motor and stress-related emotional disorders indicates that this aspect, although not directly tested in the present study, may be especially important to consider further. For example, interesting data on rats (Kametani, 1988; Kametani et al., 1984) clearly show age-related alterations in grooming activity and its behavioural microstructure. Therefore, it is possible to assume that an in-depth analysis of grooming in rats tested at different ages, using our present algorithm, may allow detection of age-related motor/coordination and affective disorders. If successful, this possibility may have an important clinical relevance, especially considering the growing number of such disorders in the elderly. Finally, given high sensitivity of rat grooming and its microstructure to various pharmacological manipulations (Barros et al., 1992, 1994; Choleris et al., 2001; D'Aquila et al., 2000; Eguibar and Moyaho, 1997), we suggest that ethologically-oriented analysis of grooming in rats, such as reported here, may be successfully used in the field of psychopharmacology research, including screening of compounds with mixed or unclear properties, or testing novel psychotropic anxiolytic and antidepressant drugs.

Acknowledgements

This research was supported by grants from CIMO Finland, the University of Tampere, the Medical Research Council of Tampere University Hospital (EVO) and the Academy of Finland.

References

Aguilar R, Gil L, Gray JA, Driscoll P, Flint J, Dawson GR, et al. Fearfulness and sex in F2 Roman rats: males display more fear though both sexes share the same fearfulness traits. Physiol Behav 2003;78:723–32.

Barros HM, Tannhauser SL, Tannhauser MA, Tannhauser M. Effect of sodium valproate on the open-field behavior of rats. Braz J Med Biol Res 1992;25:281–7.

Barros HM, Tannhauser SL, Tannhauser MA, Tannhauser M. The effects of GABAergic drugs on grooming behaviour in the open field. Pharmacol Toxicol 1994;74:339–44.

Belzung C. Measuring rodent exploratory behavior. In: Cruzio WE, Gerlai RT, editors. Handbook of molecular genetics for brain and behavior research. New York: Elsevier; 1999. p. 738–49.

Berridge KC, Fentress JC, Parr H. Natural syntax rules control action sequence of rats. Behav Brain Res 1987;23:59–68.

Berridge KC. Comparative fine structure of action: rules of form and sequence in the grooming patterns of six rodent species. Behavior 1990;113:21–56.

Berridge KC, Whishaw IQ. Cortex, striatum and cerebellum: control of serial order in a grooming sequence. Exp Brain Res 1992;90:275–90.

Berridge KC, Aldridge JW. Super-stereotypy I: enhancement of a complex movement sequence by systemic dopamine D1 agonists. Synapse 2000a;37:194–204.

Berridge KC, Aldridge JW. Super-stereotypy II: enhancement of a complex movement sequence by intraventricular dopamine D1 agonists. Synapse 2000b;37:205–15.

Bolivar VJ, Danilchuk W, Fentress JC. Separation of activation and pattern in grooming development of weaver mice. Behav Brain Res 1996;75:49–58.

Bressers WM, Kruk MR, Van Erp AM, Willekens-Bramer DC, Haccou P, Meelis E. The hypothalamus: cross-roads of endocrine and behavioural regulation in grooming and aggression. Neurosci Biobehav Rev 1998;23:163–77.

Choleris E, Thomas AW, Kavaliers M, Prato FS. A detailed ethological analysis of the mouse open field test: effects of diazepam, chlordiazepoxide and an extremely low frequency pulsed magnetic field. Neurosci Biobehav Rev 2001;25(3):235–60.

Crawley JN, Belknap JK, Collins A, Crabbe JC, Frankel W, Henderson N, et al. Behavioural phenotypes of inbred mouse strains: implications and recommendations for molecular studies. Psychopharmacology 1997;132:107–24.

Crawley JN. Behavioural phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioural tests. Brain Res 1999;835:18–26.

Cromwell HC, Berridge KC. Implementation of action sequences by a neostriatal site: a lesion mapping study of grooming syntax. J Neurosci 1996;16:3444–58.

Cromwell HC, Berridge KC, Drago J, Levine MS. Action sequencing is impaired in D1A-deficient mutant mice. Eur J Neurosci 1998;10:2426–32.

D'Aquila PS, Peana AT, Carboni V, Serra G. Exploratory behaviour and grooming after repeated restraint and chronic mild stress: effect of desipramine. Eur J Pharmacol 2000;399:43–7.

Dunn AJ, Berridge CW, Lai YI, Yachabach TL. CRF-induced excessive grooming behavior in rats and mice. Peptides 1987;8:841–4.

Eckstein RA, Hart BL. Grooming and control of fleas in cats. Appl Anim Behav Sci 2000;68:141–50.

Eguibar JR, Moyaho A. Inhibition of grooming by pilocarpine differs in high- and low-yawning sublines of Sprague–Dawley rats. Pharmacol Biochem Behav 1997;58:317–22.

Eguibar JR, Romero-Carbente JC, Moyaho A. Behavioral differences between selectively bred rats: D1 versus D2 receptors in yawning and grooming. Pharmacol Biochem Behav 2003;74:827–32.

Escorihuela RM, Fernandez-Teruel A, Gil L, Aguilar R, Tobena A, Driscoll P. Inbred Roman high- and low-avoidance rats: differences in anxiety, novelty-seeking, and shuttlebox behaviors. Physiol Behav 1999;67:19–26.

Espejo EF. Structure of the mouse behaviour on the elevated plus-maze test of anxiety. Behav Brain Res 1997;86:105–12.

Fentress JC. The tonic hypothesis and the patterning of behaviour. Ann NY Acad Sci 1977;290:370–94.

File SE, Mabbutt PS, Walker JH. Comparison of adaptive responses in familiar and novel environments: modulatory factors. Ann NY Acad Sci 1988;525:69–79.

Gerlai R, Henderson JF, Roder JC, Jia Z. Multiple behavioural anomalies in GluR2 mutant mice exhibiting enchanced LTP. Behav Brain Res 1998;95:37–45.

Greer JM, Capecci MR. Hoxb8 is required for normal grooming behaviour in mice. Neuron 2003;33:23–34.

Hart BL, Pryor PA. Developmental and hair-coat determinants of grooming behaviour in goats and sheep. Animal Behav 2004;67:11–9.

Homberg JR, van den Akker M, Raaso HS, Wardeh G, Binnekade R, Schoffelmeer AN, de Vries TJ. Enhanced motivation to self-administer cocaine is predicted by self-grooming behaviour and relates to dopamine release in the rat medial prefrontal cortex and amygdala. Eur J Neurosci 2002;15:1542–50.

Hossain SM, Wong BK, Simpson EM. The dark phase improves genetic discrimination for some high throughput mouse behavioral phenotyping. Genes Brain Behav 2004;3:167–77.

Jarbe TU, Andrzejewski ME, DiPatrizio NV. Interactions between the CB1 receptor agonist Delta 9-THC and the CB1 receptor antagonist SR-141716 in rats: open-field revisited. Pharmacol Biochem Behav 2002;73:911–9.

Kalueff AV. Measuring grooming in stress and comfort. Proc Int Conf Measur Behav 2000;3:148–9.

Kalueff AV. Grooming and stress. KSF: Kiv; 2002.

Kalueff AV, Lou YR, Laaksi I, Tuohimaa P. Increased grooming phenotype in mice lacking Vitamin D receptors. Physiol Behav 2004a;82:405–9.

Kalueff AV, Lou YR, Laaksi I, Tuohimaa P. Grooming microstructure in mice is affected by genetic ablation of Vitamin D receptors. Proc FENS Forum 2004b:353.

Kalueff AV, Tuohimaa P. Grooming analysis algorithm for neurobehavioural stress research. Brain Res Prot 2004a;13:151–8.

Kalueff AV, Tuohimaa P. Contrasting grooming phenotypes in C57BL/6 and 129S1/SvlmJ mice. Brain Res., 2004b, in press.

Kametani H, Osada H, Inoue K. Increased novelty-induced grooming in aged rats: a preliminary observation. Behav Neural Biol 1984;42:73–80.

Kametani H. Analysis of age-related changes in stress-induced grooming in the rat. Ann NY Acad Sci 1988;525:101–13.

Katz RJ, Roth KA. Stress-induced grooming in the rat – an endorphin mediated syndrome. Neurosci Lett 1979;13:209–12.

Kobayashi S, Ohta M, Miyasaka K, Funakoshi A. Decrease in exploratory behavior in naturally occurring cholecystokinin (CCK)-a receptor gene knockout rats. Neurosci Lett 1996;214:61–4.

Komorowska P, Pisula W. Does changing levels of stress affect the characteristics of grooming behaviour in rats? Int J Comp Psychol 2003;16:237–46.

Komorowska J, Pellis SM. Regulatory mechanisms underlying novelty-induced grooming in the laboratory rat. Behav Process 2004;67:287–93.

Lawler CP, Cohen PS. Pawgrooming induced by intermittent positive reinforcement in rats. Ann NY Acad Sci 1988;525:417–9.

Moody TW, Merali Z, Crawley JN. The effects of anxiolytics and other agents on rat grooming behaviour. Ann NY Acad Sci 1988;525:281–90.

Moyaho JR, Eguibar JL, Diaz A. Induced grooming transitions and open field behaviour differ in high- and low-yawning sublines of Sprague–Dawley rats. Anim Behav 1995;50:61–77.

Moyaho A, Valencia J. Grooming and yawning trace adjustment to unfamiliar environments in laboratory Sprague–Dawley rats (*Rattus norvegicus*). J Comp Psychol 2002;116:263–9.

Nasello AG, Sassatani AS, Ferreira FS, Felicio LF, Tieppo CA. Modulation by sudden darkness of apomorphine-induced behavioral responses. Physiol Behav 2003;78:521–8.

Ohl F, Toschi N, Wigger A, Henniger MS, Landgraf R. Dimensions of emotionality in a rat model of innate anxiety. Behav Neurosci 2001;115:429–36.

Osborne PG, Mataga N, Onoe H, Watanabe Y. Behavioral activation by stimulation of a GABAergic mechanism in the preoptic area of rat. Neurosci Lett 1993;158:201–4.

Perier C, Tremblay L, Feger J, Hirsch EC. Behavioral consequences of bicuculline injection in the subthalamic nucleus and the zona incerta in rat. J Neurosci 2002;22:8711–9.

Rodgers RJ, Davies B, Shore R. Absence of anxiolytic response to chlordiazepoxide in two common background strains exposed to the elevated plus-maze: importance and implications of behavioural baseline. Genes Brain Behav 2002;1:242–51.

Sachs BD. The development of grooming and its expression in adult animals. Ann NY Acad Sci 1988;525:1–17.

Salome N, Viltart O, Darnaudery M, Salchner P, Singewald N, Landgraf R, et al. Reliability of high and low anxiety-related behaviour: influence of laboratory environment and multifactorial analysis. Behav Brain Res 2002;136:227–37.

Spruijt BM, van Hooff JA, Gispen WH. Ethology and neurobiology of grooming behavior. Physiol Rev 1992;72:825–52.

Van de Weerd HA, Bulthuis RJ, Bergman AF, Schlingmann F, Tolboom J, Van Loo PL, et al. Validation of a new system for the automatic registration of behaviour in mice and rats. Behav Process 2001;53:11–20.

Van Erp AM, Kruk MR, Meelis W, Willekens-Bramer DC. Effect of environmental stressors on time course, variability and form of self-grooming in the rat: handling, social contact, defeat, novelty, restraint and fur moistening. Behav Brain Res 1994;65:47–55.

Van Erp AM, Kruk MR, Willekens-Bramer DC, Fermont PC, Nijsen MJ. PVH lesions do not inhibit stressor-induced grooming in the rat. Physiol Behav 1995;57:887–92.

Whishaw IQ, Metz GA, Kolb B, Pellis SM. Accelerated nervous system development contributes to behavioral efficiency in the laboratory mouse: a behavioral review and theoretical proposal. Dev Psychobiol 2001;39:151–70.