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Research report

The effects of chronic social defeat stress on mouse self-grooming behavior and its patterning

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ABSTRACT

Stress induced by social defeat is a strong modifier of animal anxiety and depression-like phenotypes. Self-grooming is a common rodent behavior, and has an ordered cephalo-caudal progression from licking of the paws to head, body, genitals and tail. Acute stress is known to alter grooming activity levels and disrupt its patterning. Following 15–17 days of chronic social defeat stress, grooming behavior was analyzed in adult male C57BL/6J mice exhibiting either dominant or subordinate behavior. Our study showed that subordinate mice experience higher levels of anxiety and display disorganized patterning of their grooming behaviors, which emerges as a behavioral marker of chronic social stress. These findings indicate that chronic social stress modulates grooming behavior in mice, thus illustrating the importance of grooming phenotypes for neurobehavioral stress research.

1. Introduction

Because social factors play a key role in human stress-precipitated brain disorders [1–6], social defeat stress is widely used in biomedical research to model various psychiatric disorders in animals [7–15]. Several versions of chronic social defeat stress are available for biopsychiatry research [7,16–20]. A typical social defeat paradigm evokes social confrontations between two conspecifics, in which the winner (dominant) and the loser (subordinate) animal can be identified at the end of the social interaction [6,9,21]. While acute social stress occurs after a single confrontation [16,22–25], chronic social stress requires learned social defeat over an extended period of days or weeks [3,26,27], often in combination with chronic exposure to sensory stimuli from aggressive mice [18,21,27]. Depending on the procedure, social stress induces various physiological and behavioral symptoms, ranging from anxiety to anhedonic depression, immune deficits, and altered expression of key brain genes [28–34].

Although behavioral manifestations of chronic social stress have been explored extensively [35–37], relatively little is known about its effects on animal grooming behavior. Self-grooming is an important and evolutionarily ancient behavior that is observed across many animal taxa, and constitutes 15–50% of waking time in rodents [17,27,38–41]. Beyond the primary purpose of hygiene and caring for the body surface, rodent grooming serves a variety of other functions, including stimulation of the skin, thermoregulation, chemo-communication, de-arousal, and stress reduction [17,38,40–46]. Grooming is also an intricately patterned behavior which generally proceeds in a cephalo-caudal direction, from licking the paws, to head, body, legs, genitals and tail [38,42,43,47].

Representing a common animal behavior [47–50], grooming responses to chronic social stress therefore merit further scrutiny. Several lines of evidence support this notion. For example, chronic mild stress is known to negatively affect the rodent coat state, and therefore a focus on grooming behavior clearly becomes important [7,44,51]. Although recent studies have reported the effects of acute stress on grooming behaviors [38,50,52,53], the impact of chronic stress on grooming behavior remains unclear. Since rodent grooming can be evoked and effectively assessed using exposure to novel observation chambers [48,54], here we explore the effects of chronic social defeat stress on mouse spontaneous, novelty-evoked

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self-grooming behavior and its behavioral microstructure (patterning).

2. Methods

2.1. Animals and housing

Thirty-one adult male C57BL/6J mice were included in this study. The animals were obtained from Jackson Laboratory (Bar Harbor, Maine) and acclimated for 4–6 weeks prior to testing. The animals were housed 4–5 mice/cage in the Tulane University Vivarium in Plexiglas cages (27.5 cm length, 21.5 cm height, 16.5 cm width) with standard bedding, as well as *ad libitum* access to food and water. At the beginning of the experiment, mice were 3–4 months old, and weighed 22–26 g. The animals remained on a 12:12 light/dark cycle (on: 06:00 h, off: 18:00 h) for the duration of the study. The present study adapted the sensory contact model, developed by N. Kudryavtseva's group, and currently widely accepted as a valid chronic social defeat paradigm [6,49,55]. At the beginning of the experiment, each mouse was paired with a conspecific, and housed in pairs in larger Plexiglas cages (23 cm height, 23 cm width, 44 cm length) containing a partition in the middle of the cage lengthwise. The partitions were made from transparent Plexiglas (0.5-cm thick), and each had 30 small 0.7-cm holes allowing constant sensory contact, but preventing animals from physical contact. To maximize mouse aggression, we avoided using littermates as pairs that undergo social confrontations. The housing of mouse pairs was re-arranged every 2–3 days in accordance to their social status determined by previous social defeat testing. More aggressive mice-winners were housed with less aggressive counterparts, to avoid the reduction in winners' aggression due to fighting with equally aggressive partners. Mice failing to show a clear social status by day 5 of the experiment were removed from the study.

In order to more fully compare winner and loser cohorts, we introduced an additional control group of mice ($n = 8$), housed under different conditions than the experimental groups at the beginning of the experiment. As recommended in the original chronic social defeat protocol [6,49,54], these control mice were individually housed in standard Plexiglas cages for 5 days. Such specific housing conditions were necessary for the control subjects, since their group housing would otherwise produce confounding (anxiolytic-like) effects on grooming and other behaviors assessed here. At the same time, their individual housing for 5 days was short enough to prevent unwanted anxiogenic-like modulation of all our behavioral endpoints, likely to be caused by a longer social isolation [6,49,54]. Therefore, the control group used here, displaying more standardized and less confounded 'baseline' behavior, was most suitable to be compared to both experimental groups.

2.2. Social defeat testing

Behavioral testing was performed between 12:00 and 16:00 h. Partitioned cages with mouse pairs were taken from the holding room and placed in the testing room for 15 min for acclimation (needed for animals to become awake and active). Water bottles and plastic cage tops were promptly removed, and one or two wide black lines were put on their tails by a marker, to enable their recognition by the observers. Animal handling during marking procedure was performed with great ease, in order to avoid stressing the animals. Following the "behavioral activation" period, the divider was removed for 15 min to allow for interaction between the two mice. Animal agonistic behaviors were scored by trained observers (inter-rater reliability > 0.85) based on latency (s) and frequency of sniffing, touching, dominant hetero-grooming, chasing and biting. Jointly initiated behaviors between the two conspecifics were also scored in this study (as a 0.5-score assigned to both fighting mice). In order to create a lingering scent of the opponent after the social conflict, bedding from each cage side was mixed prior to returning the partition to its original position. Tampering with their homes in this way served to irritate the mice and reinforce the induction of the social stress. Depending on levels of mouse aggression, the duration of daily social confrontations in this model may differ between the laboratories. While some groups use 10-min social confrontations [56,57] for their aggressive mice, our mice showed intermediate levels of aggression (see further), and therefore a longer 15-min duration of daily confrontation was used for our studies. After each day of interaction, a clear dominant winner mouse would typically emerge. In contrast, a passive mouse would typically display defensive behaviors, such as sideways or upright submissive postures, withdrawal, fleeing, lying on its back, or freezing. Winners ($n = 11$) were defined as mice that were dominant in $\geq$70% of their social encounters during the entire duration of social defeat paradigm as assessed by daily "win-or-lose" scoring. Losers ($n = 12$) were defined as animals experiencing only $\leq$20% of victories during the entire duration of the experiment. Mice showing similar levels of aggression were qualified as a "tie" for each social confrontation, and this outcome did not count towards the percentage of victories. Mice with predominantly unclear aggressive phenotype were not included in this study. To further confirm the categorization of the social status of the mice, an alternative "point system" method was employed, which assigned a value to each mouse depending on whether they had been judged to win, lose, or tie each social defeat encounter. Winner mice were given 3 points, each mouse involved in a tie was given 2 points, and losers were given 1 point at the end of each social confrontation. The data was then averaged for each individual animal across all 15–17 social defeat days it fought.

2.3. Novelty-evoked grooming and non-grooming behaviors

Animal novelty-evoked grooming was assessed on days 15–17 of the social defeat protocol, using the grooming analysis algorithm developed previously in our laboratory [49,50,58,59]. On the day of testing, the animals were brought to the testing room for 1 h of acclimation. Each mouse was then removed from the home cage and placed in a Plexiglas observation cylinder (diameter: 13.75 cm, height: 15 cm) with a white plastic platform underneath and a glass cover on top. The relatively small size of the cylinder was chosen for this study to minimize novelty factor that by evoking exploratory behaviors may potentially confound grooming phenotypes. Mouse behavior in this cylinder was observed manually by two experienced observers (inter-rater reliability > 0.85), and was also recorded with a digital video camera (see further), for 5 min. Non-grooming behavioral scores included the number of protected (wall-leaning), unprotected (front paws in the air), and total (protected + unprotected) vertical rears, as well as the number of freezing bouts (see further). Mice were returned to home cages at the end of testing. To eliminate the scent of previously tested mice, the testing cylinder was cleaned with 70% ethanol (vol/vol) after each trial.

For grooming behaviors, we analyzed the latency (s), frequency and duration (s) of grooming bouts. In addition to these cumulative grooming scores, behavioral patterning and regional distribution of this behavior were also assessed as described previously [47,49,58,60,61], scoring the total number of grooming episodes, the total number of transitions between episodes, the average number of transitions per bout, the average number of episodes per bout, and the percent of correct transitions. Grooming microstructure was manually recorded based on transitions to/from the paw, head, body, and tail/genital regions. Correct transitions were classified as following a complete cephalo-caudal direction, while incorrect transitions were categorized as either *skipped* (e.g., head to tail), *aborted* (e.g., head wash to no grooming), or *reversed* (e.g., body to head), as described previously [44,50]. Since regional distribution of grooming may be a useful indicator of rodent stress [42], we used the slow-motion mode of recorded videos and analyzed mouse grooming activity directed at different body parts, including paws, head, body, and tail/genitals. Rostral grooming was considered to be grooming of the paws and head, while caudal grooming included the body, legs, tail, and genitals [36].

2.3.1. Ethograms

In order to obtain a general picture of how grooming activity is embedded into other mouse behaviors, we also applied ethograms to our analyses. Adopting the approach previously used to characterize mouse phenotypes [11,62], all novelty-evoked behaviors were recorded as a sequence of the following behavioral activities: grooming, freezing, unprotected vertical rearing, wall leaning and horizontal locomotion. The ethograms were generated for each group, expressing behavioral activities as circles, and the transitions between them as arrows. Based on average mean values for each mouse group, the diameter of the circles proportionately represented the frequency of each behavioral activity. Respectively, the width of each arrow represented the number of transitions from one behavior to the other.

2.4. Computer analysis of animal behavior

To further characterize animal behaviors, we complemented manual observation with modern video-tracking tools, such as Noldus EthoVision XT6 (Noldus Information Technology, Leesburg, VA) and CleverSys Homecage Scan (CleverSys, Inc., Reston, VA) systems. In Noldus EthoVision XT6, the arena was defined to include the entire cylinder. In each trial, the arena was calibrated with the same dimensions to ensure consistency of the parameters in which Noldus EthoVision XT6 detected transitional mouse movements. Notably, this analysis was used only as an indirect 'global' measure of mouse locomotor activity (distance travelled and average velocity), complementing the endpoints obtained using more precise manual observation. To further characterize mouse behavior, CleverSys Homecage Scan recognized and automatically calculated the frequency of various behaviors, including remaining low, remaining rear up, body stretches, sniffing, digging and immobility. In both studies, video-recording was performed from the side, by a camera placed 20 cm away from the observation cylinder (i.e., in a position most suitable for video-recording of grooming behaviors).

2.5. Statistical analysis

Data calculated from the two video-tracking systems were exported to a Microsoft Excel database prior to analysis. Since a control group was not involved in social defeat testing, social behaviors were analyzed and statistically compared between the winner and the loser groups only. For grooming and anxiety-related behaviors, the control group was utilized for pair-wise comparisons with the winner or the loser experimental cohorts, respectively. All data were expressed as mean $\pm$ SEM. Behavioral differences between the groups were analyzed using a Mann–Whitney *U*-test; correlation between two social status indices was analyzed using the Spearman correlation test. Significance was set at $P < 0.05$, and a trend was noted for $P = 0.05$–0.1.

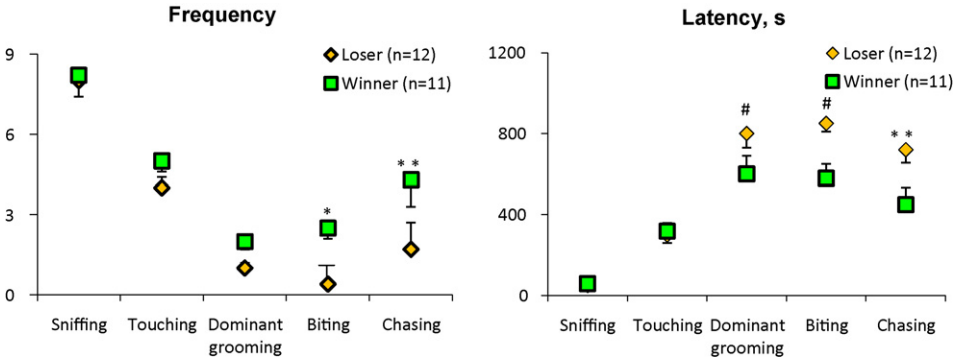

Fig. 1. Quantification of social behaviors in winner ($n = 11$) and loser ($n = 12$) C57BL/6J male mice, averaged for 15–17 days of chronic social defeat stress. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P = 0.05$–0.1 (trend), *U*-test between the two groups. Note the growing phenotypical dissection of winner and loser groups, as social aggression endpoints move from low-aggression sniffing to more aggressive biting and chasing.

3. Results

3.1. Social behavior

Quantification of animal social behaviors during the confrontations revealed marked differences between losers and winners. Overall, the winner mice won $81 \pm 7\%$ fights, compared to $5 \pm 2\%$ in the loser cohort ($P < 0.00001$, *U*-test). The point system scores were 2.8 ± 0.1 in winners vs. 1.4 ± 0.1 in loser mice ($P < 0.0001$, *U*-test). Both methods of social behavior quantification showed high correlation ($R = 0.95$, $P < 0.0001$). Winner mice initiated predictably more biting and chasing, compared to loser mice, and also exhibited shorter latencies to these behaviors (Fig. 1), generally confirming social status established based on "win-or-lose" scoring of their history of victories and losses.

3.2. Ethograms and non-grooming behaviors

Ethograms were applied here as a methodological tool [62] to analyze the sequential patterning (organization) of various behavioral events/activities and their frequencies. This approach, aiming to determine whether social stress has a global impact on mouse behavioral strategies, complemented the quantification of mouse behaviors performed by traditional endpoint-based methods (see further). Overall, the analysis of vertical, horizontal, grooming, and freezing behaviors revealed less unprotected vertical rears and transitions from horizontal to unprotected vertical activity in both experimental cohorts, fewer transitions from unprotected rears to horizontal locomotion in the winners, as well as more transitions from freezing to horizontal locomotion in the loser group (Figs. 2 and 3). Compared to control mice, transitions from horizontal locomotion to grooming were more frequent in the winner cohort, and transitions from protected vertical activity to grooming were more frequent in the loser cohort. No other significant differences were found between the groups, indicating that chronic social defeat did not result in major large-scale alterations in animal behavioral patterning.

Global assessment of mouse locomotion using Noldus EthoVision XT6 revealed decreased distance travelled and average velocity (trend in winners) in both experimental groups, compared to controls (Fig. 3). CleverSys Homecage Scan further dissected mouse behavior, revealing no overt differences between the groups in frequencies of remaining low (14–16%), remaining rear up (2–3%), body stretches (0–1%), sniffing (7–8%), digging (1–4%), and immobility (20–21%).

3.3. Traditional grooming measures

Focusing on grooming behavior and examining its "cumulative" scores, we found that both the winner and the loser groups showed somewhat similar grooming duration, frequency, and the number of episodes and transitions between episodes. The latency of grooming was not affected in the three groups (Fig. 4), collectively indicating that the traditional cumulative grooming scores are not sensitive to phenotypical differences between the three groups.

3.4. Patterning and regional distribution of novelty-induced grooming

In order to examine whether the sequencing of mouse grooming is sensitive to social stress, we calculated the percent of

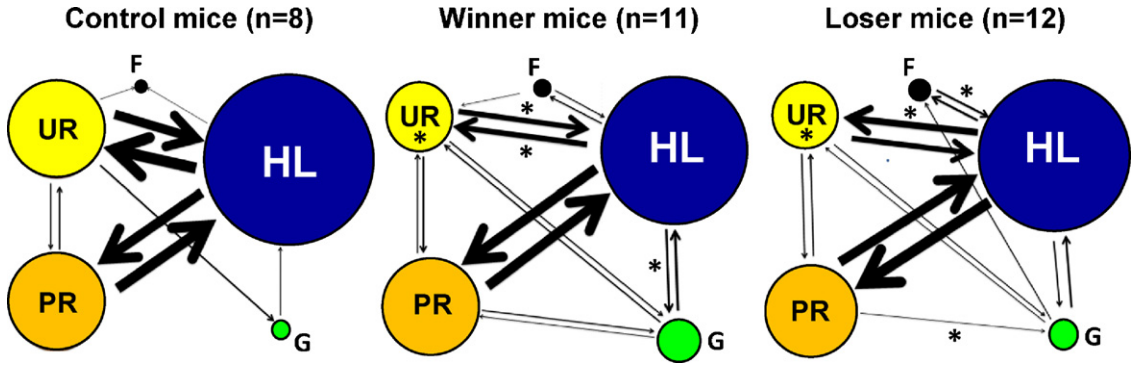

Fig. 2. Ethograms-based analysis of behavior microstructure in three groups of C57BL/6J male mice tested in the novel observation cylinder for 5 min. Circles indicate different types of behavioral activity (UR, unprotected vertical rears; PR, protected vertical rears (wall-leaning); G, grooming activity; HL, horizontal locomotion; F, freezing behavior) while arrows represent the transitions between the respective behavioral activities. The diameter of each circle and the width of each arrow indicate frequencies of individual behaviors and transitions between them, respectively. $^{*}P < 0.05$ vs. control mice, *U*-test.

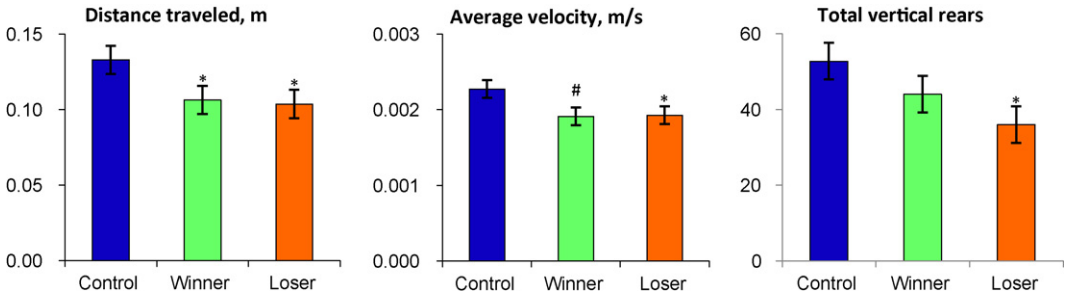

Fig. 3. Alterations in non-grooming behaviors, induced by chronic social defeat stress, in three groups of C57BL/6J male mice tested in the novel observation cylinder for 5 min. Average distance travelled and velocity data were calculated using Noldus Ethovision XT6. Vertical rears (combined protected and unprotected rears) were scored using manual observation; $^{*}P < 0.05$, $^{\#}P = 0.05–0.1$ (trend) vs. control mice, *U*-test.

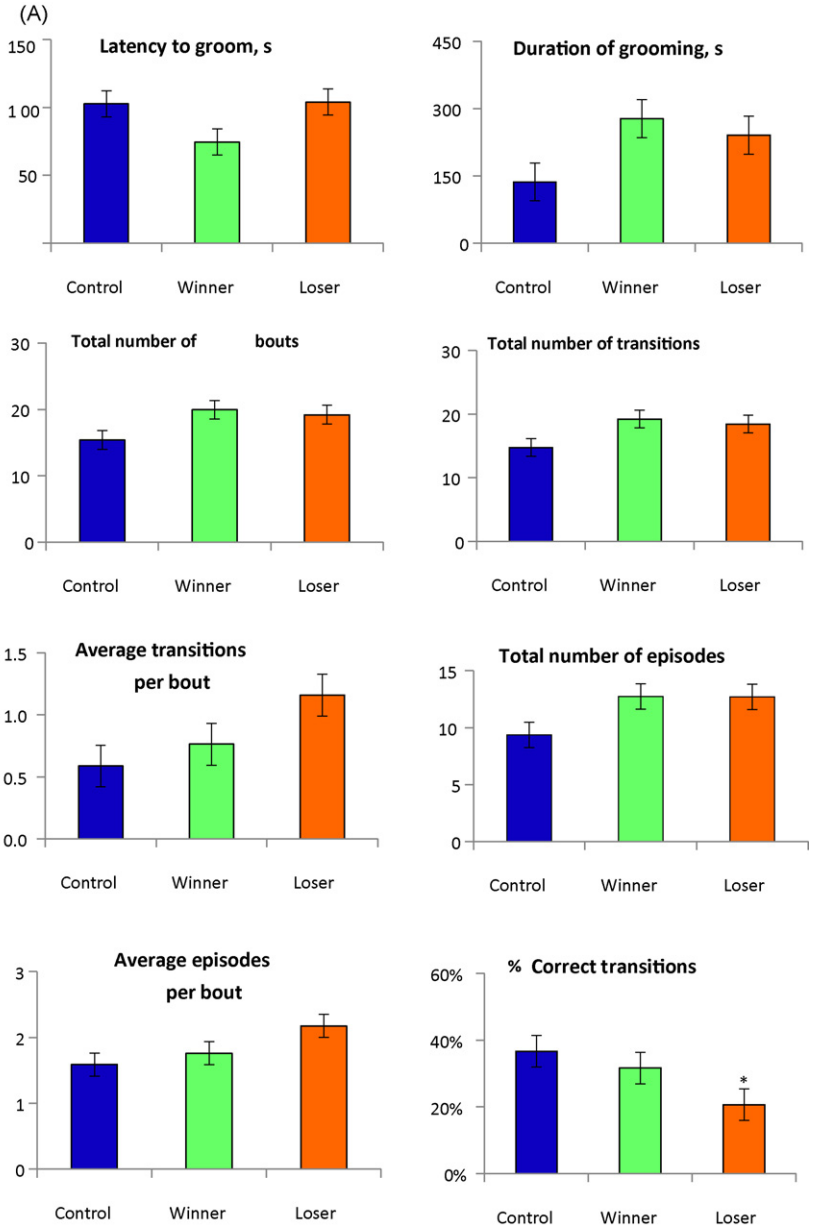

Fig. 4. Alterations in grooming behavioral endpoints induced by chronic social defeat stress in C57BL/6J male mice tested in the novel observation cylinder for 5 min. Data include both general (cumulative) and patterning endpoints (A), as well as regional distribution of grooming frequency and durations (B). $^{*}P < 0.05$, $^{\#}P = 0.05–0.1$ (trend) vs. control mice, *U*-test.

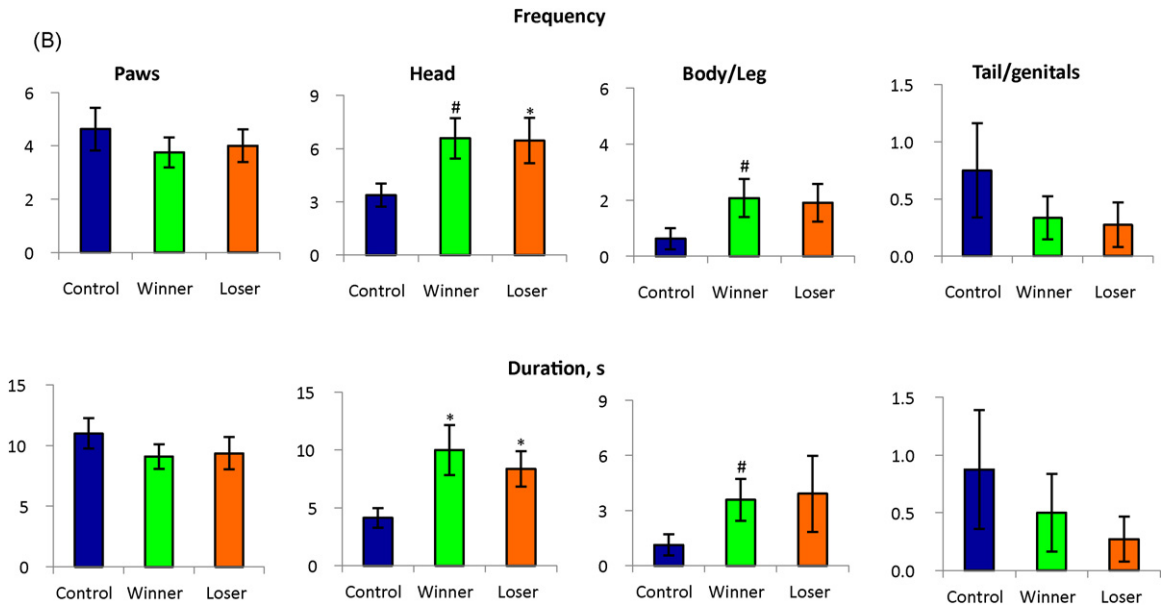

Fig. 4. (*Continued*).

correct transitions in the winner, loser, and control groups. Overall, the loser mice showed significantly fewer correct grooming transitions (Fig. 4A) compared to control mice, while the winners displayed unaltered phenotypes. Collectively, these data show less organized sequencing of grooming in the loser mice (compared to other groups), with a higher number of rapid incorrect grooming episodes, typically observed in the hyper-aroused anxious mice [50]. In contrast, characterization of incorrect transitions into skipped, reversed and aborted revealed no phenotypical differences, with approximately equal 30% distributions of transition errors of each type, in all three groups (data not shown).

Chronic stress equally affected the regional distribution of grooming activity in both winners and losers, resulting in unaltered paw and tail/genital grooming, but elevated head and body grooming, compared to their control counterparts (Fig. 4B). The duration of "rostral" head grooming, commonly associated with increased anxiety [36], was particularly higher in the loser cohort, suggesting that they had the highest levels of stress among all three groups.

4. Discussion

Self-grooming is an important animal behavior, frequently observed as a result of experimental, pharmacological and genetic manipulations [15,35,38,39,42,45–47]. Commonly seen in rodent models and tests, grooming is emerging as a useful behavioral domain to study stress-related phenotypes [35,42,63,64]. Although previous studies have shown that acute stress generally modulates rodent grooming activity (e.g., [53]) and disrupts its sequencing [36,47,48,50], relatively little is known about the effects of chronic stress, and especially chronic social stress, on mouse self-grooming behavior.

Focusing on grooming cumulative scores, several studies have attempted to address this problem. As already mentioned, chronic stress produces an overall decline in coat state, implying that grooming activity is reduced in chronically stressed mice [12,65,66]. In line with this, antidepressant treatments have been shown to reverse disheveling effects of stress [65–67]. Likewise, chronic social crowding stress inhibits frequency [68], and chronic social isolation stress reduces both frequency and duration [69] of rat grooming. However, more precise quantification of rodent grooming in chronic stress paradigms yielded rather conflicting results. For example, no effects were observed on mouse grooming activity in the open field following chronic mild stress [7,43,46]. Others reported reduced grooming frequency in young, but higher frequency in aged, rats exposed to chronic unpredictable stress [10]. Repeated ethanol injections or novelty exposure increased grooming behavior in "short sleep" mice compared to "long sleep" mice [49]. Furthermore, repeated restrain-induced stress increased open field grooming duration in rats, and although antidepressant desipramine reversed these effects, it increased grooming frequency in the chronic mild stress group [44]. Comparisons of two selectively bred mouse strains showed that chronic social contact stress, as well as repeated behavioral testing, both elevate grooming frequency in passive/non-aggressive LAL mice, but not in their stress-resistant SAL counterparts [31]. Finally, social defeat paradigm [21,28] showed elevated grooming activity in losers *during* social confrontations, which, unlike many other forms of pathological behaviors, was not corrected by diazepam [27]. The latter observations strongly suggest that altered grooming represents an essential pathological behavior evoked in mice by chronic social stress.

Mounting evidence suggests that chronic social stressors may affect not only the amount of grooming activity, but also its patterning. For example, social isolation stress in rats impaired behavioral microstructure of rat grooming [63] analyzed using our method [49]. To further explore this possibility, our present study examined the link between mouse grooming patterning and stress produced by chronic social defeat. As already mentioned, rodent grooming is an intricately patterned behavior which generally proceeds in a cephalo-caudal direction [38,42,43,47]. In our study, loser mouse behavior was most robustly affected, exhibiting a significantly lower percentage of correct grooming transitions (in the cephalo-caudal direction) compared to the controls (Fig. 4). These results suggest that chronic social stress is a powerful inducer of variances in grooming behavior, also reconfirming the sensitivity of self-grooming patterning to stress. In contrast, grooming activity levels were unaltered in both winners and losers, indicating that traditional (cumulative) grooming activity scores may lack behavioral sensitivity necessary to separate mice based on their anxiety and social status.

Since mouse social aggression includes biting behavior, it was possible to assume that self-grooming observed here has

a mechanistic explanation, for example, related to tending bite wounds. However, since the frequency of biting *per se* was rather low in this study (Fig. 1), it was unlikely to have caused overt wounds. Indeed, following social confrontations, each mouse was examined for visible wounds occurring as result of aggressive encounter. Based on our observations, only one loser mouse (8%) in the entire cohort exhibited visible bite marks in the tail region. More detailed analyses of video from this mouse, however, revealed that its grooming behavior was not directed at tail region. Moreover, compared to control mice, both winners and losers displayed high grooming scores, with winners tending to groom even more than the losers (Figs. 2 and 4; also see earlier consistent observations that defeated rats groom *less* upon return to their home cages [53]). Collectively, these data do not seem to support the link between biting and novelty-evoked mouse grooming phenotypes in socially stressed mice.

The regional distribution of grooming activity can also reflect the stress levels, since anxious rodents generally groom their rostral regions a higher frequency than their caudal regions [35,42], also see similar results obtained using our approach in a different model of chronic social stress [63]. In the present study, more anxious socially stressed subordinate mice also exhibited this phenotype, grooming their heads significantly more often and for a longer duration (Fig. 4B). In addition to grooming, other novelty-evoked behavioral endpoints confirm higher anxiety levels in subordinate mice, displaying a shorter distance travelled, fewer vertical rears, and a lower average velocity (Fig. 3).

Overall, in line with previous reports [35,70], our study showed that chronic social defeat increases anxiety in loser mice, as assessed by both manual observation and video-tracking tools. Examining the amount and sequencing (patterning) of mouse grooming, we found that while grooming activity was not robustly altered in both stressed (loser and winner) cohorts, behavioral organization of grooming was markedly impaired only in the loser cohort. The latter finding was novel, as grooming patterning has not been assessed previously in chronic social stress models. Taken together, these findings emphasize the importance of grooming in experimental models of social stress, and support the behavioral organization of rodent grooming as a phenotype sensitive to chronic social stress.

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