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Research report

Towards high-throughput phenotyping of complex patterned behaviors in rodents: Focus on mouse self-grooming and its sequencing

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ABSTRACT

Increasingly recognized in biological psychiatry, rodent self-grooming is a complex patterned behavior with evolutionarily conserved cephalo-caudal progression. While grooming is traditionally assessed by the latency, frequency and duration, its sequencing represents another important domain sensitive to various experimental manipulations. Such behavioral complexity requires novel objective approaches to quantify rodent grooming, in addition to time-consuming and highly variable manual observation. The present study combined modern behavior-recognition video-tracking technologies (CleverSys, Inc.) with manual observation to characterize in-depth spontaneous (novelty-induced) and artificial (water-induced) self-grooming in adult male C57BL/6J mice. We specifically focused on individual episodes of grooming (paw licking, head washing, body/leg washing, and tail/genital grooming), their duration and transitions between episodes. Overall, the frequency, duration and transitions detected using the automated approach significantly correlated with manual observations ($R = 0.51–0.7$, $p < 0.001–0.05$). This data validates the software-based detection of grooming, also indicating that behavior-recognition tools can be applied to characterize both the amount and sequential organization (patterning) of rodent grooming. Together with further refinement and methodological advancement, this approach will foster high-throughput neurophenotyping of grooming, with multiple applications in drug screening and testing of genetically modified animals.

1. Introduction

Self-grooming is a key evolutionarily conserved behavior observed in multiple taxa [1–4] and involved in hygiene, stress reduction and sensory stimulation [1,2,5–7]. Rodent grooming generally follows a cephalo-caudal progression (from paws/face to tail/genitals) [4,8], and is regulated by the basal ganglia and hypothalamus [9–11]. The centrally organized nature of grooming behavior makes it especially well suited to the research of complex neurobehavioral disorders, such as basal ganglia disorders [12,13], autism [14], obsessive-compulsive disorder (OCD) [15] and attention-deficit/hyperactivity disorder (ADHD) [16].

Although rodent grooming has long been assessed in behavioral, genetic and pharmacological studies [17–20], the traditional focus on 'quantity' endpoints (latency, frequency, duration) alone may not be sufficient for scientific inquiry into the neurobiology of this complex behavior. For example, rodents groom when transitioning from rest to activity [5] – a "comfort", low-stress grooming which generally follows the cephalo-caudal progression [21]. In contrast, high anxiety also evokes rodent grooming, albeit more interrupted and disorganized, as part of the stress response and hyperarousal [21]. Because such alterations may occur with or without shifts in the amount of grooming activity [22], the parallel assessment of both traditional ('quantity') and patterning ('quality') grooming endpoints becomes a necessary task in translational biological psychiatry research [5,20,23–27].

To address this challenge, we evaluated grooming phenotypes in C57BL/6J male mice. This strain was selected for this study as a common strain in neurobehavioral research [28] with relatively low anxiety and high locomotion, grooming and emotional reactivity [25,27]. Novelty exposure and misting with water, both known to evoke robust grooming responses [5,29], were used here in order to more fully characterize grooming phenotypes combining automated and manual registration methods.

Automated neurophenotyping has become indispensable in behavioral research due to the recent availability of

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high-performance software to accurately identify animal behaviors in multiple species from rodents [30,31] to zebrafish [32,33]. The advantage of automated detection is the ability to process large amounts of data more quickly (than an observer), and to allow researchers to produce more reliable, unbiased and less variable results. In the present study, we applied a custom-modified version of HomeCageScan video-recognition software [34] (CleverSys, Inc., Reston, VA) to analyze both the quantity and quality of grooming behavior. Importantly, this study aimed not to show the utility of a particular software to assess rodent grooming, but to demonstrate (as a proof of concept) a novel approach to quantify complex grooming phenotypes, which may lead to further development of high-throughput neurophenotyping tools.

2. Methods

2.1. Animals

The present study used 40 adult (approximately 5-months old) male mice of C57BL/6J strain (originally obtained from Jackson Laboratory, Bar Harbor, ME) and housed 4–5 mice per cage with free access to food pellets and water. Prior to behavioral observation (as part of animal health inspection, to assess their fur coat state and motor responses) the mice were transported from their holding room to the testing room and allowed at least 1 h for acclimation. All testing was performed at Tulane Neurophenotyping Platform between 11:00 and 15:00 to ensure uniformity throughout the experiments. Animals were individually placed in a clear observation cylinder (13 cm in diameter, 15 cm height) for observing their behavior. To assess spontaneous novelty-evoked grooming, the mice were placed in the clear plastic cylinder, video-recorded (by two side- and front-view web cameras; LifeCam Cinema HD, Microsoft Corp., Redmond, WA) and manually analyzed for 5 min (Fig. 1). For the water-induced grooming, the mice were misted with 25 °C water three times at a distance of 15 cm, and then observed and video-recorded in the clear observation cylinder for 5 min, as described above. The observation cylinder was thoroughly cleaned using 70% ethanol (vol/vol) between subjects.

2.2. Behavioral analyses

During manual scoring, two highly trained observers (inter-rater reliability >0.85, as determined by Spearman correlation) followed the Grooming Analysis Algorithm [5,20,21] to record the latency, direction and duration of each grooming bout and its constitutive episodes (paw licks, head washes, and body/leg washes), as described elsewhere [5,20,21]. A grooming "bout" was characterized as continuous self-grooming without interruption. An "episode" was identified as portion of a single bout in which the subject is grooming a specific body region (e.g., paw licks, body/leg washes), and a "transition" was defined as a progression from one grooming episode to another separate episode within a single grooming bout, according to [5,20,21].

The videos were analyzed using the HomeCageScan (CleverSys, Inc., Reston, VA) software which detects rodent movements and behaviors based on video-tracking of multiple individual body parts, posture and frequency of movements [34] (Fig. 1). While complete grooming bouts often culminate in tail and genital washes, these were not quantified in this study due to the current difficulty of distinguishing these grooming behaviors from body/leg washes within the existing software (see further).

To optimize grooming detection in our study, we applied customized settings (based on our pilot studies using this software) to detect only grooming bouts lasting longer than 3 s. This allowed us to minimize false positives associated with detection of relatively rare extra short bouts (generally representing <5% of grooming activity, own unpublished observations). To ensure reliability between detection techniques, manually scored extra-short grooming bouts were also not assessed here. The detection settings were specifically upgraded by the manufacturer for this study, enabling the software to distinguish between the different episodes of grooming and to detect the transitions between them. Recognition features which facilitated the detection of paw licking, head washing, and body/leg washing behaviors were added to the existing HomeCageScan software package specifically for this project.

As a grooming bout grows in length, it is divided by the software into a series of fixed-length segments. These segments are then individually classified into one of the preset categories (paw licking, head washing, or body/leg washing; Fig. 1). HomeCageScan uses whole body and individual body part features, as well as grooming magnitude information, during the on-going bout to perform the classification in real time. A set of rule-based tests are used to determine a likelihood value for each preset category for a given segment. The category with the highest likelihood for that episode is elected as the winner and is recorded as the software output. The program generates an output containing all of the episode classifications for a given subject at the end of each trial. Due to software constraints, a file-based output was not available at the time of this study. Therefore, researchers took a screenshot of the grooming output for each individual subject and subsequently analyzed the data for transitions. A more permanent solution to this issue will likely become accessible soon, as the field of bioinformatics is rapidly advancing.

Finally, we aimed to optimize grooming detection by comparing data from front- and side-view cameras. For this, we recorded mouse grooming using two cameras in parallel (Fig. 1), and both videos were independently recorded, analyzed and compared to establish the extent of agreement (see further).

2.3. Statistical analyses

After each video was analyzed, the produced data on the total number of grooming episodes, the duration of grooming, and the number of transitions between specific grooming episodes was compared to the manually scored data using the ranked Spearman correlation test, to establish the reliability of software-recognized vs. observer-detected scores. Automated data was also compared with manual observations using the paired Wilcoxon–Mann–Whitney *U*-test. Spontaneous vs. water-induced grooming data was compared using the *U*-test, and side-view vs. front-view video-recording data was analyzed using Spearman correlation and paired *U*-test. Statistical significance was set at $p < 0.05$ in all experiments.

3. Results

Experiment 1 examined baseline grooming and its sequencing in mice exposed to the novel environment of the observation cylinder. The comparison of manual with software-generated data revealed a significant correlation for the total number of grooming transitions (Fig. 2) and various specific transitions, including "no behavior to head washes", "head washes to paw licks" and "head washes to body/leg washes". Weaker but significant correlations were also found for "body/leg washes to head washes" and "body/leg washes to no behavior", demonstrating the utility of automated quantification of spontaneous, novelty-induced grooming behavior. The paired *U*-test found no differences between the manual and software-generated results for any of the grooming endpoints studied, confirming that automated software reliably detects specific forms of grooming behavior.

Experiment 2 tested the applicability of this approach to different types of grooming, comparing water-induced with spontaneous grooming in mice. Increased grooming activity exhibited by water-misted mice was reliably detected both by manual observers and by the automated software, including the number of total transitions (manual and automated $p < 0.05$) and specific transitions, such as "no behavior to head washes" (manual $p < 0.05$; automated $p < 0.005$) and "head washes to body/washes" (manual and automated $p < 0.05$). Manual observation revealed a significant increase in "body/leg washes to no behavior" transitions ($p < 0.05$) for the water-treated group, with a similar trend for the automated data ($p = 0.06$). Non-significant increases were detected by both methods for "head washes to paw licks" (manual NS; automated $p = 0.08$) and "body and leg washes to head washes" (manual NS; automated $p = 0.07$) in the water-treated mice (Fig. 2). Once again, the paired *U*-test revealed no significant differences within groups for software-generated data vs. manual observations (data not shown).

Finally, we compared side- and front-view video recordings to optimize the detection of grooming, finding no significant differences in the data generated by both cameras (Experiment 1; data not shown). The side-view camera detected only the small number of bouts "missed" by the front view camera (~3% of grooming bouts) due to the mouse facing away from the front camera, as data generated from both cameras appears to be essentially identical ($R = 0.92$, $p < 0.05$).

4. Discussion

Recent advances in information technology have markedly improved automated neurophenotyping in various animal models [33,35–38]. This study was the first report to apply behavior recognition-based video-tracking tools to rodent self-grooming behavior and its patterning. Experiment 1 showed the effectiveness of our approach in identifying various grooming episodes

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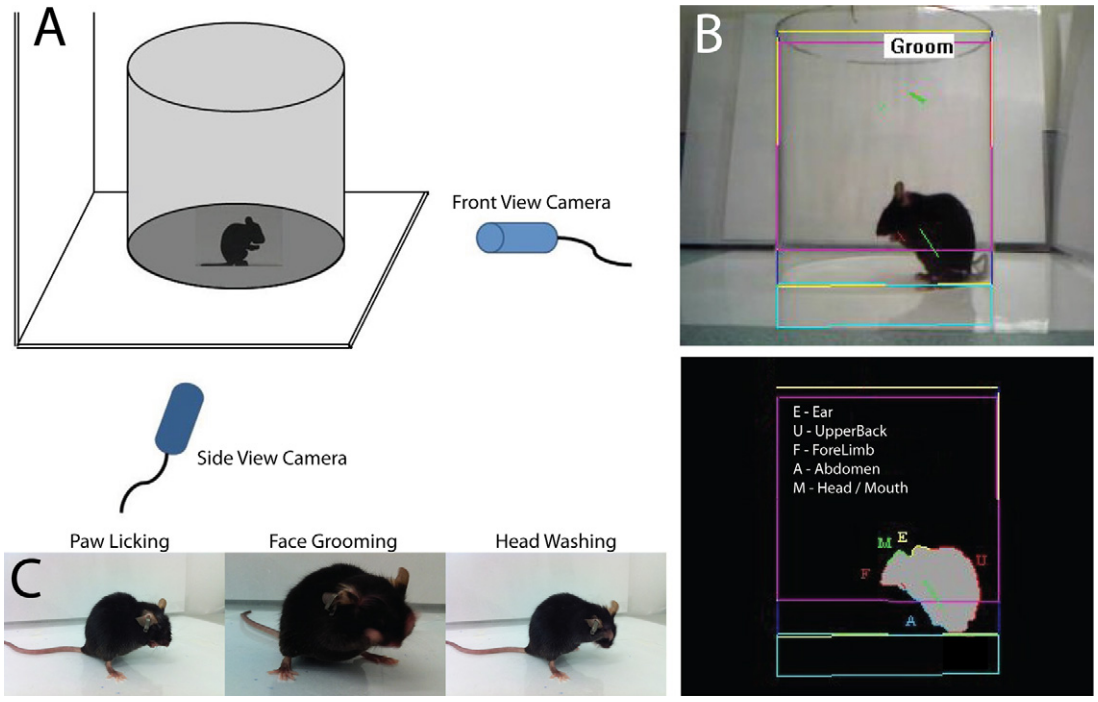

Fig. 1. Experimental set up (observation cylinder and front/side view cameras), HomeCageScan video-tracking and typical grooming behaviors observed in this study. (A) The experimental set-up used in this study. (B) An example of HomeCageScan analysis. (C) Photos representing common grooming behaviors observed in this study.

and transitions between them (Fig. 2). Experiment 2 proved that behavior-recognition software cannot only identify global changes in grooming behavior but can also characterize the microstructure (patterning) of this key animal behavior under variable conditions (Fig. 3). For example, mice misted with water displayed a systemic increase in all grooming behavior, including global and sequencing endpoints. Furthermore, water-treated mice showed a more disorganized grooming (e.g., initiating bouts with head washing) deviating from the established sequence, which typically begins with paw licking. Water-treated subjects also displayed more "backwards" sequencing (e.g., "head washes to paw licks" or "body/leg to head washes") as detected by both manual and automated recognition techniques. Collectively, these results indicate that high-throughput neurophenotyping of animal self-grooming and its sequential organization is possible based on recent behavior recognition tools.

This study is important because it opens new opportunities to study various motor and affective disorders. For example, we showed that software can easily and accurately detect differences in grooming activity and patterning (Fig. 2). This will help identify crucial elements modulating stereotypic behavior and may be used to study complex behavioral disorders such as OCD, ADHD, Tourette syndrome, autism and schizophrenia.

In addition, there were several limitations in this study. As already mentioned, tail and genital grooming was not quantified here due to the current difficulty of distinguishing these behaviors from body/leg washes in the HomeCageScan program. However, this limitation will likely be addressed once more sophisticated video-tracking methodology becomes available. Again, our goal was not to develop software as a ready-to-use solution for grooming research – rather, we aimed to prove that high-throughput automated quantification of grooming and its patterning is possible in principle.

False positives and negatives represented another problem, albeit common with any animal model. In the present study, $17 \pm 2\%$ of the grooming bouts detected automatically were false positives, and $2 \pm 0.5\%$ of all behaviors were false negatives. However, such limitations do not negate the applicability of our method, since both control and experimental groups displayed similar percentages of misdetection. Nevertheless, further methodological

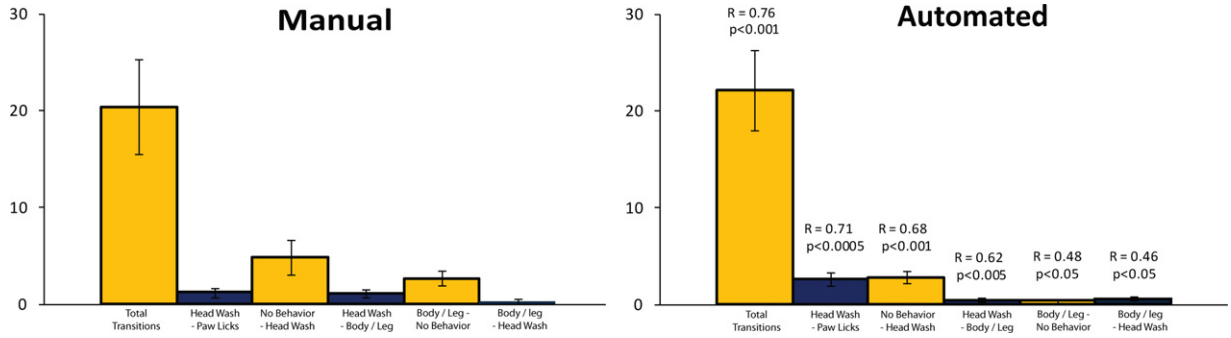

Fig. 2. Correlations of manual and automated novelty-evoked self-grooming endpoints in male C57BL/6J mice (Experiment 1). Significant positive correlations were found between manual observations and HomeCageScan data using the Spearman rank correlation test, demonstrating the utility of this approach for behavioral research ($n = 20$ in each group).

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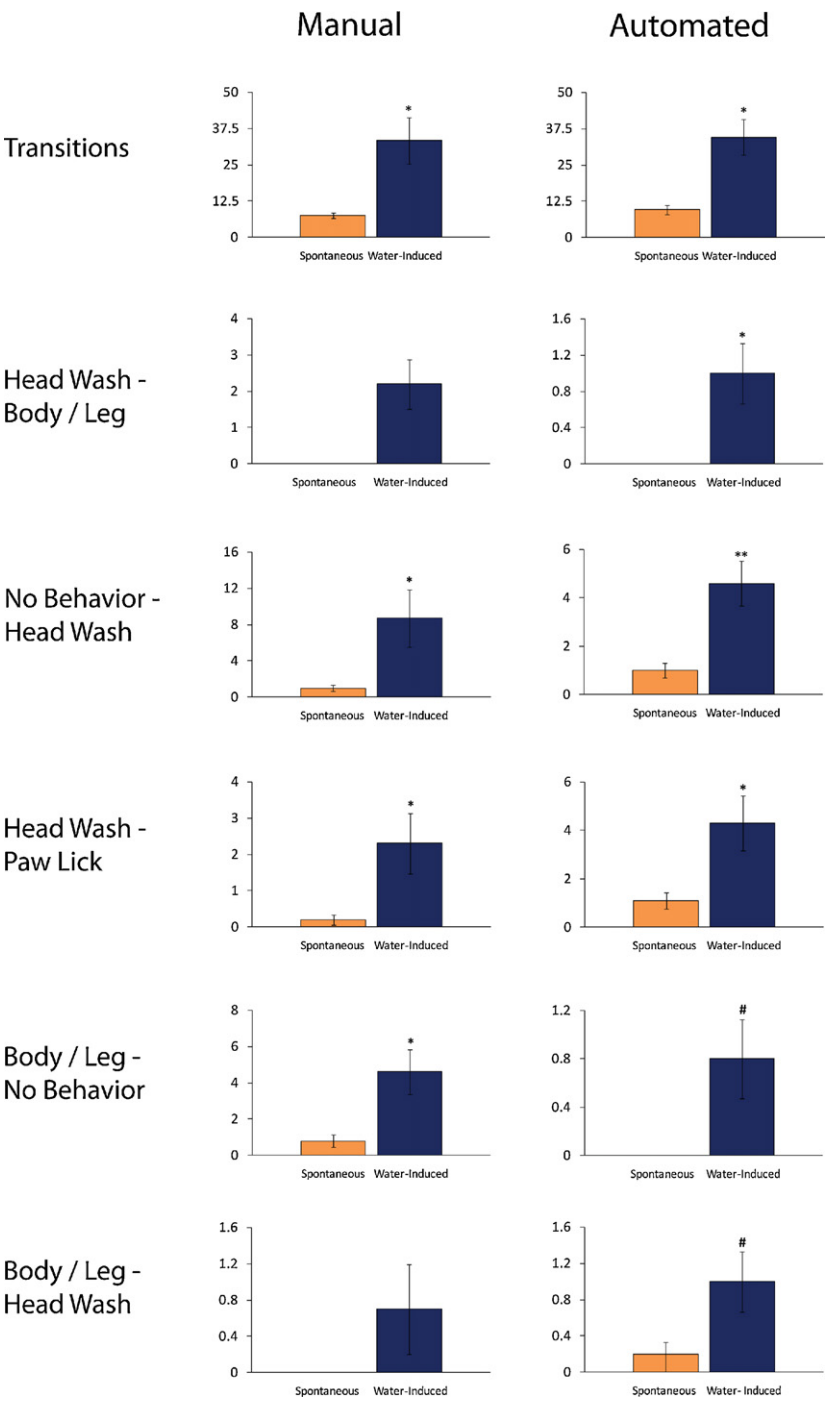

Fig. 3. Behavioral differences between total number and specific individual transitions detected by manual observation (left column) and automated video-tracking software (right column) for spontaneous (novelty-evoked) vs. water misting-evoked self-grooming in male C57BL/6J mice (Experiment 2). Notice the similarities between manual and HomeCageScan grooming analysis ($n = 10$ in each group, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{\#}p = 0.05$–0.1, trend; U-test). There were no significant differences between the respective types of grooming detected by manual and automated methods, confirming the ability of video-recognition software to reliably detect mouse grooming phenotypes.

advances in video-tracking technologies are expected to minimize false detection, improving the effectiveness of the proposed approach.

Moreover, the recording of grooming behavior from multiple angles was also addressed in this study. Analysis of camera-specific data yielded no significant differences between cameras, establishing significant correlation between the two data sets. This finding suggests that, when using currently available video-tracking tools to analyze grooming behavior, single front-view camera may be sufficient for generating consistent and reliable results in our experimental setup (Fig. 1). However, with the advent of new information technologies, future software tools could utilize the integration of signals from multiple videos within a single digital file, generating a 3D output of rodent grooming behavior (similar to the automated 3D-based detection of zebrafish swimming [33]). Such enhanced dimensionality of rodent grooming data may eventually lead to more sophisticated measures of grooming activity, markedly improving the detection of its various subtypes. Equally important, the same approach may theoretically be applied in general to any other (i.e., non-grooming) behaviors, especially several

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complex behaviors that, like grooming, follow a specific fixed pattern (e.g., courtship/mating behaviors, aggression, barbering and stereotypies).

Furthermore, our method can be applied to other biomedical problems, including the high-throughput assessment of self-grooming behavior to identify differences in motor activity/control or emotionality in various inbred, mutant and transgenic strains [5,24,25]. For example, mice lacking the pituitary adenylate cyclase-activating polypeptide gene display abnormally high grooming [39], making this strain particularly well-suited for grooming analyses. Other strains of interest include the Hox8 knockout mice [40], and the BTBR strain [41], both displaying abnormal repetitive grooming. Our laboratory is currently performing grooming analysis on serotonin transporter (SERT−/−) and brain-derived neurotrophic factor (BDNF+/−) knockout mice to evaluate the changes in motor activity and grooming behavior evoked by these genetic mutations.

Finally, the application of our approach is not limited to mouse self-grooming behavior, and can be employed to study other animal behaviors. For example, video-tracking tools already have the ability to track multiple animals per arena [34], making the detection of hetero-grooming (which may assist in studying social phenotypes [42,43]) a likely possibility with applications for autism research. Our approach is also not limited to mice and is expected to work with other species, such as rats, due to marked cross-species similarities in grooming behavior [5,20,21]. Moreover, grooming behavior is not only exhibited by rodents and is common in various species including nonhuman primates [1,44,45] and humans [46–48]. Although they may show considerable differences in self-grooming techniques and syntax, the general conceptual and methodological framework outlined here can be adapted for the study of other model organisms. Another promising application of our approach, enabled by the availability of video-tracking behavior-recognition technologies, may be 24 h video-recording of animal self- and hetero-grooming in more natural environments (e.g., homecages), eliminating additional stressors which may confound behavioral data.

Overall, the comprehensive analysis of mouse self-grooming activity and its patterning offers apparent benefits for the field of neurobehavioral research. The understanding of self-grooming behavior and its correlates will help elucidate the complexities of motor patterning and the neural substrates which drive repetitive behaviors. Given the sensitivity of mouse grooming to various genetic and pharmacological manipulations, this approach will enable researchers to more accurately detect objective changes in both grooming activity and sequencing, thereby advancing the field of neurophenotyping research.

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