Analyzing grooming microstructure in neurobehavioral experiments

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Published online 11 October 2007; doi:10.1038/nprot.2007.367

Grooming is a commonplace, robust behavior in rodent species. It has been shown to be highly sensitive to a number of experimental factors, making it an ideal target for manipulation. The complex patterning of grooming in rodents, which usually proceeds in a cephalo-caudal direction and involves several distinct stages, can be dissected into its constituent parts and microstructures. Several grooming patterning analysis methods are described in the protocol that allow for an assessment of this behavior based on measurements of grooming activity and its sequencing. Additionally, grooming can be evaluated in reference to the regional distribution and syntax in which it occurs. Owing to the ever-increasing number of rodent models that have strong grooming phenotypes, this high-throughput in-depth analysis is becoming crucial for biomedical research.

INTRODUCTION

Grooming behavior is common in rodents, representing up to 30–50% of their waking time[1,2]. This evolutionarily ancient behavior is frequently seen in various rodent behavioral tests[3–5], sometimes being among the most robustly affected domains[5–10]. In experiments, grooming activity can be triggered by various manipulations, such as novelty or predator stress, misting with water, or injection of different drugs and hormones[11–15]. Together with the growing number of mutant or transgenic mice with robust grooming phenotypes[16,17], this emphasizes the importance of thorough analysis of grooming behavior in biomedical research.

Traditionally, grooming activity has been assessed by measuring the latency, duration and frequency scores, sometimes providing important insights into neurobiological mechanisms[2,5,7,8,14–16,18]. However, there is a growing understanding that simply assessing the 'amount' of animal grooming may be insufficient for correct data interpretation and analysis[3,4,13,19]. Earlier studies have established a complex patterned nature of rodent grooming[20–24], which proceeds in a cephalo-caudal direction and consists of several distinct, easily distinguishable stages (**Fig. 1**). In line with the growing interest of researchers in the behavioral patterning (microstructure) aspect of grooming[25–29], quantification of grooming patterning (which is the focus of this protocol) becomes a useful approach in neurobehavioral research.

Mounting evidence indicates that grooming patterning in rodents is highly sensitive to stressors and other factors. Dissectible at a number of levels, ranging from individual limb kinematics[30] to action syntax[23,24,27,31], such complex patterned structure makes grooming particularly attractive for experimental research.

Overall, there are two different approaches to the analysis of rodent grooming patterning that will be summarized in this protocol. One approach is based on the grooming analysis algorithm (GAA) developed for behavioral characterization in rats and mice[4,13,22]. This algorithm examines all registered grooming behaviors globally, assessing their overall adherence to cephalo-caudal progression and evaluating other characteristics of grooming activity, such as interruptions and regional distribution. Using this method, we found that stressors usually disorganize cephalo-caudal grooming patterning and increase the percentages of incorrect transitions and incomplete or interrupted bouts, also disrupting regional distribution of grooming[4,13,32].

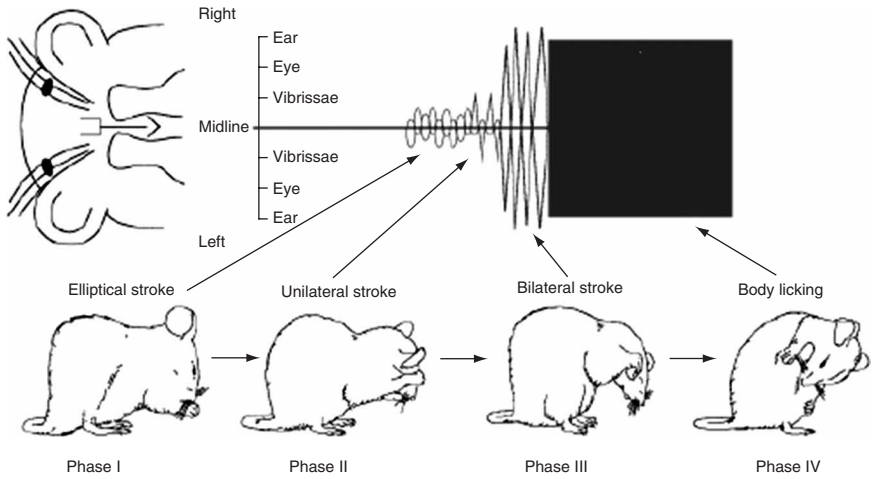

Figure 1 | Syntactic grooming chain pattern in mice (adapted from ref. 27, with permission; also see **Supplementary Videos 2** and **3** online). Phase I: series of elliptical strokes tightly around the nose (paw, nose grooming). Phase II: series of unilateral strokes (each made by one paw) that reach up the mystacial vibrissae to below the eye (face grooming). Phase III: series of bilateral strokes made by both paws simultaneously. Paws reach back and upwards, usually ascending high enough to pass over the ears (head grooming). Phase IV: body licking, preceded by postural cephalo-caudal transition from paw/head grooming to body grooming. (Note that tail/genital grooming is frequently seen in rodents as a part of cephalo-caudal grooming pattern, but is not presented here as it does not form a syntactic chain.)

Another important approach focuses on syntactic aspects of grooming, splitting this behavior into flexibly ordered mixtures of strokes, licks or scratches (non-chain grooming; **Supplementary Video 1** online) and fixed syntax patterns (chains; **Supplementary Video 2** online)[33–35]. A typical grooming chain is imbedded in other forms of grooming behavior and serially links up to 25 grooming movements into four predictable phases that follow one syntactic rule[27,31] (**Fig. 1**, **Supplementary Videos 2** and **3** online). Phase 1 consists of 5–9 rapid elliptical strokes over the nose and mystacial vibrissae lasting for about 1 s. Phase 2 is short (0.25 s) and consists of small asymmetrical strokes of increasing amplitude. Phase 3 consists of large bilateral strokes that take 2–3 s for the animal to complete. The chain concludes with phase 4, which consists of a postural turn followed by a period (1–3 s) of body licking directed to the flank. This last phase varies more in length than other phases and often ends by blending into subsequent non-chain grooming in which chains are embedded. For practical purposes, the signature, rapid, elliptical strokes of phase 1 provide the best marker for the stereotyped syntactic chain.

The serial structure of chains is more repetitive and consistent in order and time, compared to flexible, non-chain grooming. The special nature of syntactic chain grooming is highlighted by dopamine D1 receptor activation, which produces a relative enhancement of chain over non-chain grooming, and complementary reduction by D2 agonists[25,26]. Using this approach in mice and rats, numerous studies have shown that brain lesions[36–38], pharmacogenic stimulation[25,26,28], genetic ablation of brain receptors[39] and stressors[29] lead to altered grooming sequencing, manifest in altered percentages of grooming chain initiation and/or completion.

In general, as will be discussed further in more detail, there are clear benefits of an in-depth patterning-oriented assessment of grooming behavior of rodents. First, it allows a better focus on the grooming domain *per se*, with a potential utility to mimic obsessive-compulsive disorder (OCD) and other similar behavioral disorders. Second, grooming patterning emerges as a sensitive index of altered animal anxiety and emotionality. Third, it has sensitivity to various physiological, pharmacological and genetic manipulations. Taken together, this makes analyses of rodent grooming based on this protocol an important tool in biomedical research.

MATERIALS

REAGENTS

- Laboratory mice or rats. Most mouse strains listed in the Mouse Phenome Project (http://www.jax.org/phenome) and many mutant mice listed in Mouse Genome Informatics databases seem suitable, although grooming activity varies markedly between the strains and may be confounded by neurological and other specific phenotypes **! CAUTION** Experiments must follow national and institutional guidelines for the care and use of laboratory animals (see ref. 40 for details on housing, husbandry and handling).
- Drugs of choice: saline (used as a vehicle in most cases); anxiolytic or anxiogenic drugs; antidepressants. The most frequent routes are intraperitoneal, intramuscular or subcutaneous. Pretreatment time varies depending on the activity of the drugs and the route of administration.

EQUIPMENT

- Small transparent observation boxes or cylinders (e.g., 20 cm × 20 cm × 30 cm for mice; 30 cm × 30 cm × 30 cm for rats) **! CAUTION** Between sessions, the apparatus has to be thoroughly cleaned with 30% (vol/vol) ethanol, to remove olfactory cues.

PROCEDURE

Acclimation

1| Transfer rodents to the procedure room (for acclimation) 1 h before testing.

Induction of grooming response

2| Remove the rodent from the cage and expose it to a stress that will induce grooming. For example, novelty stress—exposure to an unfamiliar observation box for 5 or 10 min—usually evokes high levels of spontaneous grooming activity[19,41]. Stronger stressors (e.g., a 5-min pre-exposure to predator or bright light) will elicit robust grooming responses in most cases[4,19,32]. In addition to spontaneous grooming, this behavior can be induced artificially (e.g., following misting rodents with water (using spray), making them swim or smearing them with food[28,42]). Misting with water is the easiest procedure to induce such grooming. For this, place the rodent on the experiment table, lifting it slightly by the tail (mice) or holding gently by the scruff of the neck (rats) to minimize movements. Use a standard spray bottle filled with pure water at room temperature (20 °C, use 'misting' and not 'stream' mode). Face the rodent toward the direction of the nozzle (25–30 cm away) and spray three times to adequately coat the animal with mist. Place the rodent into the observation box. The procedure does not require pretraining or special handling of animals.

? TROUBLESHOOTING

Grooming analysis algorithm

3| Use a timer to record general cumulative measures of grooming activity, such as the latency to onset, the duration and number of grooming episodes (bouts). Assess the average duration of a single grooming bout (calculated as total time spent grooming divided by the number of bouts). Also record grooming patterns for each bout: paw licking, nose/face grooming (strokes along the snout), head washing (semicircular movements over the top of the head and behind ears), body and leg grooming/scratching (body fur licking and scratching the body with the hind paws) and tail/genitals grooming (licking of the genital area and tail). In addition, assess the total number of transitions between grooming stages and the average number of transitions per bout (calculated as the total number of transitions divided by the number of bouts). The following scaling system can be used to analyze grooming microstructure, based on grooming stages defined in **Figure 1** (with modifications): no

grooming (0), paw licking (1), nose/face/head wash (2), body grooming (3), leg licking (4) and tail/genitals grooming (5). Analyze grooming interruptions and correct versus incorrect transitions between patterns. Correct transitions between grooming stages include the following progressive transitions: 0–1, 1–2, 2–3, 3–4, 4–5 and 5–0. Incorrect transitions are chaotic and are characterized by skipped (e.g., 0–5, 1–5, etc.) or reversed (e.g., 3–2, 4–1, 5–2, etc.) stages. Four main types of incorrect transitions include aborted (prematurely terminated, e.g., 3–0, 4–0), skipped (e.g., 1–5, 2–5), reversed (e.g., 3–2, 4–1, 5–2) and incorrectly initiated (e.g., 0–4, 0–5). Three main ethological measures of grooming patterning include the percentage of incorrect transitions (of total transitions) and the percentages of interrupted and incomplete grooming bouts. The percentages of incorrect transitions can also be easily calculated for each of the four subcategories separately. In addition, the duration of correct versus incorrect patterns and complete versus incomplete bouts can be calculated. A 'complete' bout consists of the following sequence of patterns: 0–1–2–3–4–5–0; all other bouts should be considered 'incomplete'. A grooming bout is considered 'interrupted' if at least one interruption was recorded within its transitions. Interruptions greater than 6 s determined separate grooming bouts. Assess the regional distribution of grooming, as directed to the following five anatomical areas: forepaws, head, body, hind legs and tail/genitals. Rostral grooming includes forepaw (preliminary rostral grooming) and head grooming. Body, legs and tail/genital grooming can be arbitrarily considered as caudal grooming. Calculate (i) the percentage of total grooming patterns, (ii) the percentage of time spent grooming and (iii) the interruptions, which can be calculated for each anatomic area (as well as analyzed in rostral versus caudal aspects). Categorize each grooming bout as being directed to (i) multiple regions or (ii) a single region, and calculate the percentage of grooming bouts and the percentage of time spent grooming for both categories[4,32,43,44]. The procedure does not require pretraining or special handling of animals. **Figure 2** shows typical results seen in rodents using GAA-based behavioral analyses of grooming sequencing.

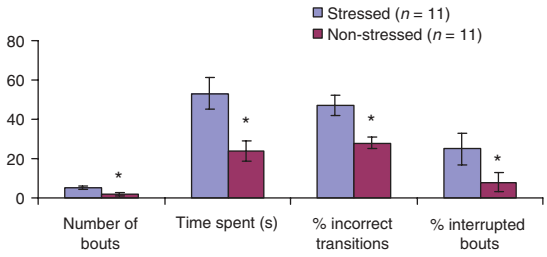

Figure 2 | Example of stress-evoked alterations in grooming sequencing in rats detected using the GAA (rats have been stressed by exposure to a brightly illuminated novel environment for 5 min; $*P < 0.05$, *U*-test).

? TROUBLESHOOTING

Grooming syntax analysis

4| Handle the animals and put them in the recording chamber for a short period of time (10–30 min) 3–4 d before study, for acclimation to the testing environment and filming procedures.

5| Remove the rodent from the cage and transfer it to the recording chamber.

6| Place the rodent in a recording chamber with a clear plastic floor and a video camera to record from below. Use a frame-by-frame offline analysis of the videotapes, using both a choreographic notation system developed for detailed descriptions of stereotyped grooming sequences (**Fig. 1**) and a computer-assisted scoring system (that transcribes the occurrence of each grooming stroke, lick or other movement, as well as limb trajectory amplitude and laterality, and other movements such as rearing, stepping, head turning and reaching)[25,26,33].

? TROUBLESHOOTING

7| Assess the number of initiated syntactic chains, the number of fully completed chains (through phase IV in **Fig. 1**), the probability of chain initiation (the number of initiated syntactic chains per minute of grooming time) and the probability of pattern completion once initiated (the percentage of fully completed grooming chains of total number of initiated chains)[25,26,28]. A common method for cataloging behavior relies on sampling behavior at regular intervals (e.g., 15 s). As flexible non-chain grooming dominates normal grooming with syntactic chains interspersed irregularly and infrequently at rates of about 5–10 chains per 2-h session in normal, undisturbed animals (our unpublished observations), observation periods and recordings of 1–2 h are typical minimum durations necessary to expose the syntactic chain and its properties. Videotapes should be analyzed in slow motion (one-tenth of actual speed) independently by two trained observers blind to the groups (compare scores by different observers and re-score the tapes to obtain consensus on each measure). **Figure 3** shows typical results seen in rats using the syntactic chain-oriented analysis of grooming sequencing. In general, sampling methods have clear advantages in many applications. More animals can be studied per session, as a single observer can scan multiple cages. In contrast, continuous tabulation relies on tediously examining all movements one by one. Sampling methods are particularly productive in identifying the basic elements of grooming behavior and the effects of drug manipulations on grooming behavior[45–48].

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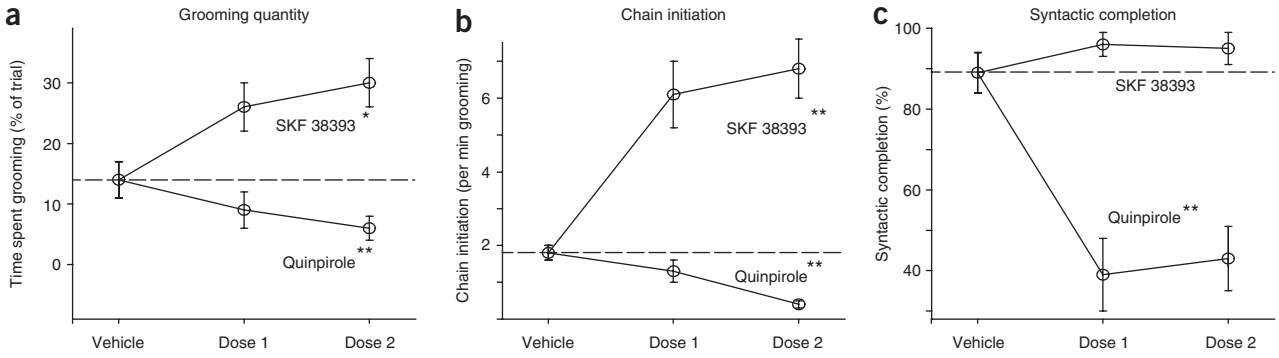

Figure 3 | Example of drug-evoked alterations in grooming sequencing in rats detected by the syntactic chain analysis approach. Effects of dopamine D1 (SKF 38393) and D2 (quinpirole) agonists on stereotyped grooming are shown (adapted from ref. 26). (**a**) Amount of grooming as a percentage of total time spent grooming during entire observation period. (**b**) Syntactic chain initiation as the number of syntactic chains per minute of grooming behavior. (**c**) Syntactic completion as percentage of grooming chains that were fully completed, as a proportion of those that were begun. Dose 1: SKF 38393 = 10 mg kg^{-1}, quinpirole = 5 mg kg^{-1}. Dose 2: SKF 38393 = 20 mg kg^{-1}, quinpirole = 10 mg kg^{-1}. *$P<0.05$. **$P<0.01$.

Non-grooming behaviors

8| In addition to rodent grooming, assess non-grooming behaviors, such as exploration, motor coordination and motor activity. Grooming behavior is generally highly sensitive to baseline anxiety, fatigability, as well as musculoskeletal, vestibular or neurological abnormalities. To rule out these factors, a battery of tests assessing these phenotypes[42,49] may be necessary, to complement grooming-oriented research.

? TROUBLESHOOTING

Data analysis

9| Use the Mann–Whitney *U*-test for comparing two groups (parametric Student's *t*-test may be used if data are normally distributed) or an analysis of variance (ANOVA) for multiple groups, followed by a *post hoc* test. More complex designs, such as one-way ANOVA with repeated measures (time) or *n*-way ANOVA (additional factors: treatment, genotype, stress, sex, etc.), can also be used in grooming studies.

● TIMING

5–10 min for GAA and 1–2 h or more for grooming syntax analysis

? TROUBLESHOOTING

Step 2: low grooming activity

Some animals may display abnormally low grooming activity. In general, this may represent (a) overall strain inactivity (if so, re-assess if the strain is suitable, to avoid the ceiling/floor effect (depending on which way the stimulus is likely to drive a change)), (b) neurological/vestibular phenotype (consider additional behavioral testing for motor/coordination and balancing[49]) or (c) strain-specific 'low grooming' phenotypes[3] (re-assess if the strain is suitable, but may be interesting to examine). However, in other cases, this may be due to high initial anxiety that can be reduced by improving handling procedures and using less stressful testing conditions (reduced illumination, smaller boxes, etc.). It is important to use relatively small observation boxes, to reduce novelty-evoked anxiety and minimize exploratory and other confounding behaviors. Always acclimate animals for at least 1 h before the testing.

Step 2: high grooming activity

Some animals may show abnormally high stereotypic-like grooming activity. In some cases, this may be strain-specific phenotype with a strong OCD-like component[16,17,50] that merits further scrutiny. In some cases, this may reflect overall stress in the animal facility, and improved husbandry/enrichment[40,51,52] would help normalize animal behavior. The same applies to the problem of high individual and group variability of grooming patterning that may also arise in experimental analyses of grooming.

Step 2: role of prior experience

As many studies nowadays involve batteries of tests, it is important to consider potential effects of test batteries on grooming and its sequencing. A minimum 7-d acclimation period may be necessary to minimize potential confounds in grooming patterning studies (such as habituation).

Step 2: context-specific grooming
Some forms of grooming are context-specific and require additional consideration. For example, body scratching with hind legs in rodents may not be temporally integrated with other grooming elements. Likewise, males during mating will generally display genital licking, which is a part of sexual behavior (rather than grooming activity *per se*). Thus, separate registration and analysis of these context-specific forms of grooming may be necessary to avoid risks of misinterpreting behavioral experimental data.

Step 3: interrupted bout versus two separate bouts
Grooming interruptions, frequently seen in rodent behavior, can pose some difficulties for the registration and interpretation of data. How long should one wait before an interruption can be concluded as the end of a bout? In our studies, an interruption of more than 6 s was recorded as the termination of the bout. If the grooming resumed at any point after the 6-s cutoff, it was recorded as the initiation of a new bout. Using an arbitrary, reasonable (i.e., long enough) and consistent cutoff time period can help avoid confusion.

Step 3: atypical (rare) forms of grooming
Some strains may show peculiar, relatively rare and strain-specific types of grooming, such as 'vertical' grooming[41] or extra-short bouts of 'pre-grooming'[53] that are difficult to classify. However, these additional forms of grooming may be a useful source of biobehavioral information, and their quantification may be advisable.

Step 6: data collection
The use of event recorder/timer and video recording grooming data is necessary, for better detection and accuracy. The use of frame-by-frame analysis of the syntactic chains will be necessary to improve the quality and overall reliability of the detection of grooming patterns.

Step 7: missing grooming syntax by sampling methods
Although sampling methods are very extensive and of high-throughput, they have some disadvantages for reconstructing detailed sequential organization of patterns. Unless the time between samples is extremely short (i.e., effectively continuous sampling), the detailed temporal structure of grooming may be missed. For example, to detail the multiple strokes of phase 1 or phase 2 transition in syntactic grooming chains (**Fig. 1**), sampling intervals would need to be less than a few video frames (1/30 s per frame). Owing to the similarity of chain and non-chain grooming strokes, the special sequential properties of grooming chains may be missed by sampling methods. To detect the order of grooming actions, all strokes must be cataloged and observed for at least 1–2 h, to more fully expose the syntactic chain and its properties.

Step 7: very few syntactic grooming chains
During a 2-h session, most rodents will produce enough (ten or more) grooming chains to examine their patterning. However, some manipulations (e.g., D2 agonists) may lead to fewer syntactic grooming chains (2–3 per hour). As ten or more chains are usually required for behavioral analyses, a longer observation time (e.g., 3–4 h) may be necessary to obtain enough chains.

Step 8: prior test history
As grooming activity and its patterning may be affected by prior testing history, it may be suggested to perform such tests as a mini-battery after the initial grooming patterning study.

Step 8: combining two experiments in one
An alternative approach may be to study non-grooming behaviors simultaneously with grooming behavior in the same experiment. For example, monitoring horizontal and vertical exploration, as well as defecation and urination, in the observation box (representing a small open field) can be an informative index of animal motor activity and emotionality, to parallel grooming data. Likewise, when using swim-induced grooming, swimming behavior (to control for motor activity) and post-swim vertical rears (to control for fatigability[42]) may be examined, in addition to assessing grooming.

ANTICIPATED RESULTS

Rodent grooming has two notable sequence patterns: non-chain sequences in more variable and flexible patterns (**Supplementary Video 1** online) and a fixed chain sequence[33–35] (**Fig. 1**, **Supplementary Videos 2** and **3** online). Both grooming patterns are composed of the same actions—only the rigidity of the sequence structure differs. Stereotyped chain grooming is less frequent (2–15 chains per hour) comprising a total of approximately 10–75 s of grooming. In contrast, non-chain grooming is more frequent, with total durations up to or more than 20 times greater than chain grooming.

It is expected that rodent grooming patterning (assessed by the GAA and grooming syntax-oriented analyses) may be sensitive to various experimental manipulations, with several important practical applications. For example, grooming patterning

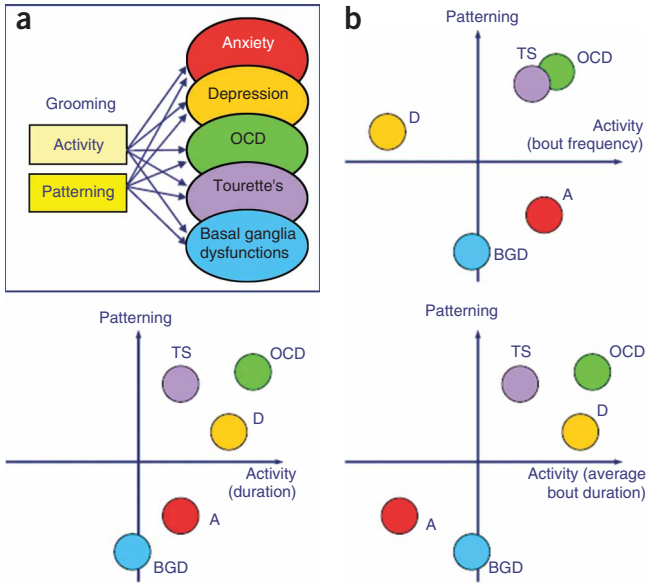

Figure 4 | Potential applications of grooming activity and patterning analyses to behavioral research. (**a**) Relevance of grooming indices to modeling selected human brain/behavioral disorders (these disorders are given as examples only). (**b**) Activity-patterning plotting of grooming data (small colored circles) and their relevance to corresponding disorders in **a** (A, anxiety; BGD, basal ganglia dysfunctions; D, depression; OCD, obsessive-compulsive disorder; TS, Tourette's syndrome), expected to be seen in a genetic model (e.g., a novel mutant mouse strain) in question.

scores presented here may be used for examining mouse and rat stress- and emotionality-related behaviors[4,29,32,41] (**Fig. 2**; also see **Fig. 4**) or behavioral effects of various drugs. Whereas the GAA showed that grooming microstructure in mice and rats is sensitive to acute anxiogenic, anxiolytic[13,32], antidepressant[54] and chronic anxiolytic[55] drugs, assessment of grooming syntax revealed increased rigidity of its patterning to dopaminergic drugs (such as D1 agonists)[25,26] and disorganized sequencing of grooming following administration of D2 agonist quinpirole[26] or repeated subchronic phencyclidine[28].

Additionally, analysis of grooming patterning (e.g., grooming syntax) becomes an indispensable tool in brain lesion studies that explore the role of various brain structures (especially basal ganglia) in motor control and the regulation of patterned behaviors[33,34]. Moreover, grooming microstructure emerges as a source for ethologically oriented experimental models of human stereotypic behavioral disorders, such as OCD or Tourette's syndrome[27] (**Fig. 4**). Finally, addressing the need for new reliable protocols to assess rodent phenotypes, grooming patterning analyses are becoming useful for behavioral neurogenetics, showing their sensitivity to mutation-evoked alterations in grooming sequencing[27,44] as well as genotype differences among selected mouse strains[4,41].

In general, more attention needs to be paid to evaluating grooming patterning in experimental models and to further development of analytical software that will be able to detect microstructure of grooming. Among several different computerized videotracking tools, microbehavior recognition-based approaches seem to be particularly promising for developing fully automated grooming analytic systems. It is expected that high-throughput extensive analyses of grooming microstructure will foster further translational research in the field of biological psychiatry and experimental neuroscience, assisting researchers in correct data interpretation and selecting appropriate rodent models for their research. Likewise, the addition of grooming patterning data to the extensive rodent online databases (e.g., http://www.jax.org/phenome) may foster further behavioral research, enabling a more full characterization of rodent phenotypes and generation of new grooming-based behavioral models[56].

Note: Supplementary information is available via the HTML version of this article.

ACKNOWLEDGMENTS This research was supported in part by the Medical Research Foundation (EVO) of Tampere University Hospital (Finland), the Intramural Research Program of the National Institute of Mental Health (USA) and NARSAD YI Award (to A.V.K.). We thank Professor Kent Berridge (University of Michigan, USA) for permission to use grooming syntax diagram and for his valuable comments on earlier versions of this manuscript.

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