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ABNORMAL BEHAVIORAL ORGANIZATION OF GROOMING IN MICE LACKING THE VITAMIN D RECEPTOR GENE

Allan V. Kalueff ^a; Yan Ru Lou ^a; Ilkka Laaksi ^a; Pentti Tuohimaa ^a ^a Department of Anatomy, Medical School, University of Tampere, Tampere, Finland

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ABNORMAL BEHAVIORAL ORGANIZATION OF GROOMING IN MICE LACKING THE VITAMIN D RECEPTOR GENE

ALLAN V. KALUEFF YAN RU LOU ILKKA LAAKSI PENTTI TUOHIMAA

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

Vitamin D is a steroid hormone with several important functions in the nervous system. Numerous human and animal data link alterations in the vitamin D system to various behavioral disorders. Grooming is an important element of rodent behavior with a general pattern of cephalocaudal progression (paw licking – nose/face wash – body wash – tail/genitals wash). Here we studied whether genetic ablation of vitamin D nuclear receptors (VDR) in mice may be associated with altered behavioral sequencing of grooming. Overall, VDR null mutant mice showed abnormal grooming, including a higher percentage of "incorrect" transitions and longer duration of "incorrect" grooming (contrary to the cephalocaudal progression); a higher percentage of interrupted grooming bouts; and the atypical regional distribution of grooming (more leg grooming, less body and tail/genitals grooming), compared to their wild-type controls. Grooming of heterozygous mice was similar to the wild-type group,

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Address correspondence to Allan V. Kalueff, Department of Anatomy, Medical School, University of Tampere, Tampere 33014, Finland. E-mail: avkalueff@inbox.ru

indicating that abnormal grooming patterning is inherited as a recessive. In contrast, behavioral sequencing of another complex behavior (mating with a female) was unaltered in all three genotypes, suggesting grooming-specific abnormal sequencing in these mutant mice. Our results suggest that a neurosteroid vitamin D and VDR may play an important role in controlling sequencing of grooming in mice, and further confirm the important role of the vitamin D system and VDR in the regulation of behavior.

Keywords: Nuclear receptor knockout; Behavioral sequences; sexual behavior; null-mutant homozygotes and heterozygotes

INTRODUCTION

Neurosteroid hormone vitamin D plays an important role in the nervous system including differentiation, regulation of Ca²⁺ homeostasis, modulation of neurotrophins release, and activity of key brain genes and enzymes of neurotransmitter metabolism (Carswell, 1997; Garcion et al., 2002). The functions of vitamin D are mediated through the nuclear vitamin D receptor (VDR), a member of the nuclear receptors superfamily of ligand-activated transcription factors (Kato et al., 1999). VDR is a 50-60 kDa protein, consisting of several functional domains responsible for ligand and DNA binding, heterodimerization, nuclear localization and transcriptional activation (Table I). VDR are widespread in the brain and the spinal cord including the areas involved in the regulation of motor activity and behavior (Prufer et al., 1999; Langub et al., 2001). Moreover, several clinical and experimental studies outline the possible role of vitamin D and VDR in the regulation of various behaviors (Altemus et al., 1987; Carswell, 1997; Burne et al., 2004a,b; Kalueff et al., 2004b,c).

Self-grooming is an ancient innate behavior, directed to the outer body surface, and represented across most animal species (Sachs, 1988; Spruijt et al., 1992). Biological functions of grooming include care of the body surface, removal of ectoparasites, thermoregulation, chemocommunication, sensory stimulation of skin, displacement, arousal minimization, and social interaction (Spruijt et al., 1992). Grooming is a particularly important part of the rodent behavioral repertoire (Sachs, 1988; Aldridge & Berridge, 1998; Greer & Capecci, 2003) sensitive to a number of endogenous and exogenous manipulations (van Erp et al., 1994; Kruk et al., 1998; Kalueff, 2002). In rodents, it is a complex

Domains	Structure	Functions
A/B (≈21 aa)	Hyper-variable amino-terminal domain unusually short compared to many other nuclear receptors	Lacks transactivation potential, but possibly determines the overall transactivation capacity of VDR
C (≈66 aa)	DNA-binding domain (similar to that of other nuclear receptors) with two zinc fingers interacting with specific DNA sequences on the target gene	DNA binding, also participates in nuclear localization and dimerization with the retinoic acid X receptor (RXR)
E, or E/F (≈310 aa)	Ligand-binding domain (similar to other nuclear receptors, especially RXR); organized in 13 α -helices and 3 β -sheets forming a hydrophobic pocket. Also includes the ligand- dependent activation function-2 (AF-2, \approx 18 aa, a short amplipatic C-terminal α -helix)	A multifunctional globular domain mediating selective interactions of VDR with the hormone, other nuclear receptor partners (e.g., RXR) and co-modulatory proteins (CP)*. AF-2 is crucial for ligand-activated transcription
D (≈30 aa)	Highly flexible "hinge" region	Confers rotation flexibility between C and E domains to allow simultaneous VDR dimerization and DNA-binding

Table I. Domain structure of the vitamin D nuclear receptor (VDR)

See Ahonen (2002), Ylikomi et al. (2002), and Sulton & MacDonald (2003) for details. aa—amino acids. *includes SRC family, DRIP multiprotein complex, and several other proteins interacting with and modulating the VDR transactivation/transrepression.

hierarchically organized behavior, with a general pattern of cephalocaudal progression known to proceed as follows: paw licking, nose and face wash, head wash, body wash and fur licking, leg licking, tail/genitals nitals licking and wash (Berridge et al., 1987; Sachs, 1988; Berridge & Whishaw, 1992).

There is a growing body of literature suggesting the evolutionary link between grooming behavior and the vitamin D system. Since vitamin D is synthesized in the skin after sun exposure and was found in body grease and fur (Carpenter & Zhao, 1999), certain animal behaviors, such as self-grooming, may represent important physiological adaptations of the organism in respect to regulation of the vitamin D system. This evolutionarily significant link has led to an interesting hypothesis as to why abnormal grooming might be observed in animals with impaired VDR (Kalueff et al., 2004a). Furthermore, high concentrations of VDR were recently found in the basal ganglia, brain stem, hypothalamus and the limbic system (Veenstra et al., 1998; Prufer et al., 1999; Langub et al., 2001; Walbert et al., 2001), the brain areas all known to be involved in neural control of grooming and its sequencing (Grill & Norgen, 1978; Kruk et al., 1998; Strazielle & Lalonnde, 1998; Meyer-Luehmann et al., 2002). In addition, vitamin D has been reported to be involved in VDR-mediated modulation of brain neurotransmitters, including acetylcholine and dopamine (Carswell, 1997; Garcion et al., 2003), known to regulate grooming (Cromwell et al., 1998; Kalueff, 2002). Taken together, these data suggest that the vitamin D system and VDR may be important for the regulation of grooming behavior and its sequencing.

Genetically targeted animals provide a powerful tool for neurobehavioral research, and several studies have already focused on behavioral organization of grooming in different mutant mice (Coscia & Fentress, 1993; Bolivar et al., 1996; Cromwell et al., 1998; Strazielle & Lalonde, 1998; Greer & Capecci, 2003). Mice with genetically impaired VDR are currently available for biomedical research focusing on the biological functions of vitamin D and VDR (Kallay et al., 2001; Song et al., 2003). Cloning and sequencing of the VDR gene (≈ 65 kb) has been carried out in some species including chickens, humans, rats and mice (Kamei et al., 1995). Structurally, the mouse VDR gene shows significant homology between these species: the most conserved fragments encode C (100% homology with rat and human VDR gene) and E domains (89-96% homology with human and rat VDR gene) of the receptor (Kamei et al., 1995). Several groups have independently generated VDR null mutant mice (NM) by targeted disruption of this gene. Yoshizawa et al. (1997) ablated exon 2 encoding the first zinc finger of the DNA-binding domain, while Li et al. (1997) ablated a fragment spanning exons 3-5 and encoding the second zinc finger. In addition, Erben et al. (2002) have recently generated mice expressing non-functional VDR without the first zinc finger. Since the absence of functional VDR results in the target tissue insensitivity to vitamin D, the analysis of the behavior of these mutant mice seems to be an important tool to assess the role of the vitamin D/VDR system in the brain.

Our recent studies have shown that a VDR null mutation generated in Tokyo (Kato et al., 1999) leads to abnormal grooming in mice, including increased grooming frequency and duration (Kalueff et al., 2004a). These findings further strengthen the link between vitamin D, VDR and grooming. However, various mutations have been reported to affect rodent

grooming "activity" measures, sequencing of grooming or even both, implying different degrees of motor/behavioral anomalies (Coscia & Fentress, 1993; Cromwell et al., 1998; Strazielle & Lalonde, 1998; Greer & Capecci, 2003). In addition, it has been shown that grooming patterning is sensitive to the level of stress in rodents, such as mice (Kalueff & Tuohimaa, 2004) and rats (Komorowska & Pellis, 2004). Together, this indicates that a detailed ethological analysis of grooming and its sequencing represents an important part of behavioral phenotyping of mutant mice, allowing us not only to characterize their grooming per se, but also further assess their possible motor or emotional phenotypes. Therefore, in the present study we wanted to study the role of the vitamin D/VDR system in the regulation of behavior by examining the impact of VDR genetic ablation on the behavioral microstructure (organization) of grooming in mice.

MATERIALS AND METHODS

Animals

Vitamin D receptor null-mutant (NM) mice were bred in the University of Tampere from the strain initially generated in the University of Tokyo (Yoshizawa et al., 1997). Subjects were homozygous (-/-) NM, heterozygous (+/-) HZ and homozygous (+/+) wild type (WT) 129S1 mice. Mice of all three genotypes were littermates produced by 4–5 heterozygous crosses and born in a ratio of approximately 1:2:1 (WT, HZ, NM). On Day 10 postpartum, tail clips were taken for genotyping performed using Polymerase chain reaction (PCR) on DNA prepared from tail tissue. Four primers were used to amplify a 130 bp VDR band and a 150 bp Neo band from the targeted gene. On Day 21 postpartum, pups were weaned and assigned to different cages based on their genotype and gender. Adult male mice used in the present study were 24-30 weeks old, maintained in a virus/parasite-free facility and exposed to a 12-h light, 12-h dark cycle. Lights were turned off at 18.00 PM and on at 6.00 AM. The animals were experimentally naïve and housed in groups of four in individual transparent cages ($26 \times 12 \times 14$ cm), with food and water freely available.

Apparatus and Procedures

All experiments were performed between 14.00 and 18.00 h. In the present study we examined two types of grooming behavior: (i) spontaneous

(novelty-induced) and (ii) "artificial" (swimming-induced). In our first experiment we assessed spontaneous novelty-induced grooming in the WT and NM animals (n = 12 in each groups). For this, the mice were placed individually in a clean plastic observation box $(30 \times 30 \times 30 \text{ cm})$ and observed by an experienced investigator (intra-rater reliability >0.90) for a period of 5 min, timing the duration and number of grooming patterns. In order to asses "artificial" swim-induced grooming, the mice were placed individually in a water tank for 20 s. The tank was a 30-cm glass cylinder 25 cm in diameter, filled with water $(24 \pm 1^{\circ}C)$ to a depth of 15 cm. After a short swimming session, the mice were removed from the tank, placed individually in the observation box and observed by the investigator for a period of 5 min, timing the duration and number of grooming patterns. Since our previously published studies found no behavioral difference between HZ and WT mice tested in a battery of tests (Kalueff et al., 2004b,c), and because our preliminary observations have shown that both groups display similar grooming frequency and duration, in a separate experiment we assessed their novelty-induced grooming patterns (n = 8 in each group; we did not assessed water-induced grooming and the regional distribution of grooming in these mice).

Since abnormal sequencing may be seen not only for grooming, in the third experiment we analysed behavioral patterning of a non-grooming complex behavior (mating) in all three genotypes. For this, following a 10-day acclimation time, 6 mice of each genotype used in the previous experiments, were tested in the actometer test. To induce sexual behavior, we paired sexually naïve males with a WT female in estrus, assessing the following patterns for 10 min: 1) non-sexual behavior (NSB, any behavior, not oriented toward a female, except genital self-grooming); 2) female's genital sniffing (GS), 3) follow (F), 4) mounting (with or without intromission; M) and 5) genital self-grooming (GG, genital licking after M). Both latency and frequency measures were analysed for each pattern. In addition, we analysed the transitions between these patters organized in the following sequences/bouts: (NSB-GS-NSB), (NSB-GS-F-NSB), (NSB-F-M-NSB), (NSB-GS-F-M-NSB) and (NSB-GS-F-M-GL-NSB). The percentages of each sequence (of total number of sequences) were calculated for all genotypes.

Between subjects, each apparatus was thoroughly cleaned (wet and dry cloths). All animal experiments were performed in full compliance with the European legislation on animal experimentation (86/609/EEC) and approved by the Ethical Committee of the University of Tampere.

Grooming Behavioral Analysis

General Measures. Three ethological measures of grooming activity were evaluated in all these tests: frequency (the number of grooming bouts); total time (s) spent grooming, and average duration of a single grooming bout (s) calculated as total time spent grooming divided by the number of bouts.

Patterns. The following 6-point scaling system (Kalueff & Tuohimaa, 2004) was used in the present study to assess grooming microstructure (patterns organized in bouts): no grooming (0), paw licking (1), nose and face wash (2), head wash (3), body grooming (4), leg licking (5), and tail/genitals grooming (6). Interruptions longer than 5 s determined separate grooming bouts. Additionally, each grooming pattern was categorised as being (i) "correct" (adhered to the cephalocaudal progression 0-1-2-3-4-5-6-0) or (ii) "incorrect" (contrary to this progression). The duration of incorrect patterns of both categories was assessed in this study, and the percentage of time spent "incorrect" grooming was calculated for all three genotypes.

Transitions. Transitions between grooming patterns were assessed using the grooming analysis algorithm as described earlier (Kalueff & Tuohimaa, 2004). Briefly, "correct" transitions adhered to the cephalocaudal progression as follows: (0-1), (1-2), (2-3), (3-4), (4-5), (5-6), and (6-0); "incorrect" transitions included all other possible transitions between grooming patterns. The number (total, and incorrect) of transitions and average number of the respective transitions per bout (calculated as the number of transitions divided by the number of bouts) were evaluated in this study. Correct/incorrect grooming transitions were analysed using the transition matrix, and the percentages of incorrect transitions were calculated for all three genotypes.

Bouts. A grooming bout was considered "interrupted" if at least one interruption was recorded within its transitions. A grooming bout was considered "complete" if the following sequence of patterns was recorded: 0-1-2-3-4-5-6-0. All other bouts were considered "incomplete." Complete/incomplete and interupted/uninterrupted grooming bouts were analysed in this study, and the percentages of incomplete bouts and interrupted bouts were calculated for all genotypes.

Regional Distribution. In order to assess the regional distribution of grooming in the WT and NM groups, we analysed grooming activity directed to the following anatomic areas: forepaws and head, body, hind-legs and tail/genitals. The percentage of grooming patterns, the percentage of time spent grooming, and the percentage of interruptions were calculated for each area. In addition, each grooming bout was categorised as being directed to (i) multiple regions or (ii) a single region. The percentage of grooming bouts and the percentage time spent grooming were calculated for both categories.

Data Analysis. All results are expressed as mean \pm S.E.M. Behavioral data were analysed by the Mann-Whitney U-test for independent samples. A probability of less than 0.05 was considered statistically significant.

RESULTS

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Figure 1 shows spontaneous and artificial grooming activity of the WT compared to the NM mice. Overall, in line with our previous findings (Kalueff et al., 2004a), in all test situations the NM spent more time on grooming, showing more bouts, and more transitions between stages, than did their WT littermates. Analysis of average duration of a single bout and average transitions per bout showed no significant difference between the genotypes (average bout duration: novelty-induced grooming $(9.9 \pm 1.9 \text{ s} \text{ WT}; 6.6 \pm 2.2 \text{ s} \text{ NM})$; swim-induced grooming $(9.3 \pm 2 \text{ s} \text{ WT}; 5.0 \pm 1.5 \text{ s} \text{ NM})$; transitions per bout: novelty-induced grooming $(3.6 \pm 0.7 \text{ WT}; 4.8 \pm 1 \text{ NM})$; swim-induced grooming $(6 \pm 2 \text{ WT}; 6 \pm 1 \text{ NM})$.

However, the behavioral microstructure of grooming was significantly altered in these mice (Fig. 1). A detailed ethological analysis using the grooming analysis algorithm (Kalueff & Tuohimaa, 2004) applied to both types of grooming activity revealed that mutant mice generally show a higher percentage of incorrect transitions (novelty-induced grooming: $43 \pm 6\%$ WT; $67 \pm 5\%$ NM, p < 0.05, U-test; swim-induced grooming: $25 \pm 6\%$ WT; $44 \pm 7\%$ NM, p < 0.05, U-test). Most frequent incorrect transitions of both types of grooming activity in this group included head-leg (25–34%), no grooming-leg (20–25%), leg-no grooming (10–15%), leg-head (10–15%), and body-head (10–13%), compared to no grooming-bogy (57–60%), body-no-grooming (25–31%), no

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Figure 1. Spontaneous (novelty-induced) and artificial (swim-induced) grooming. These behaviors were compared in 5-min actometer tests of VDR null mutant mice (NM) and their littermates homozygous for the normal (WT) allele of this gene. Data are expressed as mean \pm S.E.M. *p < 0.05, compared to the WT control group (U-test). NB – number of grooming bouts; GD—duration of grooming bouts (sec); TT—total number of transitions between grooming stages; % IT—percentage of incorrect transitions (of TT); % IB—percentage of interrupted bouts (of total NB).

grooming-tail/genitals (10-14%) and head-tall/genitals (8-10%) in the WT mice. Interestingly, head-leg and leg-head transitions were almost lacking in the WT group.

As can be seen in Figure 2, the mutant mice showed a trend to more average incorrect transitions per bout. Overall, the NM group demonstrated longer incorrect grooming duration (novelty-induced grooming:



Figure 2. Microstructure of spontaneous (novelty-induced) and artificial (swim-induced) grooming. Behaviors of VDR null mutant mice (NM) were compared with those of their littermates homozygous for the normal (WT) allele of this gene–assessed in the actometer test for 5 min. Data are expressed as mean \pm S.E.M. *p < 0.05, compared to the WT control group (U-test). TB—number of transitions per bout; ITB—number of incorrect (contrary to the cephalocaudal progression) transitions per bout; IGD—duration of incorrect grooming (sec).

 25 ± 4 s ($46 \pm 5\%$) NM; 10 ± 2 s ($31 \pm 5\%$) WT; p < 0.05, U-test); swim-induced grooming: 23 ± 4 s ($39 \pm 6\%$) NM; 6 ± 1 s ($17 \pm 3\%$) WT, p < 0.05, U-test). Analysis of grooming bouts (Fig. 1) shows that the NM group also demonstrated more interrupted bouts (noveltyinduced grooming: $21 \pm 3\%$ WT; $38 \pm 4\%$ NM, p < 0.05, U-test; swim-induced grooming: $14 \pm 3\%$ WT; $32 \pm 5\%$ NM, p < 0.05, U-test), compared to their WR littermates.

Table II shows the regional distribution of grooming activity in both groups of mice. Analysis of these data shows that in the WT group, novelty-induced grooming was directed most often to the forepaws and head. Other groomed areas, in rank order, were: body, hindlegs and tail/genitals. Swim-induced grooming was distributed similarly to the novelty-induced grooming, showing, however, more gradual cephalocaudal progression (Table II).

In contrast, the NM group showed different regional distribution of novelty-induced grooming, including significantly higher frequency of forepaws and head grooming, a trend to more time spent grooming this area, significantly higher frequency and duration of hindlegs grooming,

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Table II. Regional distribution of grooming activity

				Body a	reas			
	Forepaws	s and head	Bc	dy	Hin	dlegs	Tail and {	genitals
Grooming characteristics	ΜT	MN	ΤW	MN	WT	MN	WT	MN
Novelty-induced grooming								
%grooming patterns	50 ± 5	$72 \pm 4^*$	23 ± 4	$7\pm1^*$	15 ± 2	$21\pm3^{*}$	12 ± 2	•0
%time grooming	48 ± 6	65 ± 7	32 ± 4	$16\pm1^{*}$	10 ± 2	$19\pm3^*$	10 ± 1	•0
%interruptions	78 ± 5	84 ± 11	10 ± 3	4 ± 1	9 ± 2	12 ± 2	3 ± 1	0
Swim-induced grooming								
%grooming patterns	63 ± 5	73 ± 7	22 ± 5	12 ± 3	12 ± 2	15 ± 2	3 ± 1	0
%time grooming	54 ± 7	$81\pm6^*$	36 ± 5	$7\pm1^{*}$	5 ± 1	$12 \pm 1^*$	5 ± 1	•0
%interruptions	83 ± 7	72 ± 8	12 ± 4	8 ± 2	5 ± 1	20 ± 6	0	0
- GOV MN on the TW	in tantan II.	$f_{abc} = 10$ is $h_{abc} = h_{abc}$	~ Q *	0.05 /TT 4224)				

WT-wild type, NM-VDR null mutant mice (n = 10 in each group). *P < 0.05 (U-test).

and significantly lesser frequency and duration of body grooming. Interestingly, tail and genitals grooming was not observed in this group (Table II). Swim-induced grooming was organized similarly to the novelty-induced grooming, including significantly longer grooming of forepaws, head and hindlegs, a trend to higher frequency of grooming directed to these areas, as well as significantly lesser body grooming frequency and duration (p < 0.05, U-test). As can be seen in Table II, tail and genitals grooming was also lacking in this group. Moreover, the regional distribution of interruptions failed to reveal any significant difference between both groups of mice, since interruptions were predominantly associated with forepaws and head grooming in all our experiments.

The lack of tail/genitals grooming in the NM mice resulted in 100% incomplete grooming bouts, compared to a slightly lesser percentage in the WT group (88–97%). Overall, the WT mice dedicated 76 \pm 10% of novelty-induced grooming bouts and 82 \pm 8% of time spent grooming to multiple-body regions, compared to 55 \pm 7% and 63 \pm 9% in the NM group, respectively (p < 0.05, U-test). For swim-induced grooming, the NM group dedicated 35 \pm 8% of grooming bouts and 57 \pm 11% of time spent grooming to multiple-body regions, compared to 45 \pm 9% and 67 \pm 11% in the WT group, respectively (although this difference did not reach significance).

Figure 3 shows spontaneous grooming activity in the WT compared to the HZ mice. Overall, all grooming activity measures as well as the behavioral organization of novelty-induced grooming were unaltered in these mice, indicating that grooming phenotypes are almost identical in the WT and HZ animals.

Table III summarizes data on the behavioral patterning of sexual behavior in the WT, HZ and NM groups. Overall, there was no significant difference between the NM and their HZ and WT controls in these measures, including both activity scores and the behavioral microstructure. These results indicate that the sequencing of this complex behavior is not impaired in the NM or HZ mice.

DISCUSSION

Creation of the NM (Yoshizawa et al., 1997; Kato et al., 1999) had provided new insights into the role of VDR in different tissues (including brain), enabling studies of the role of the vitamin D/VDR system in

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Figure 3. Spontaneous (novelty-induced) grooming and its microstructure. Behaviors of wild-type (WT) mice and their littermates that were heterozygous for the VDR null mutation (HZ) were assessed in the actometer test for 5 min. Data are expressed as mean \pm S.E.M. p > 0.05 between groups (U-test) for all measures. Abscissa labelings as in Figs. 1 and 2.

	WT (n = 6)	HZ (n = 6)	NM (n = 6)
Behaviors	Number	Latency	Number	Latency	Number	Latency
Number of patterns						
Female genital sniffing (GS)	$17~\pm~3$	14 ± 4	$17~\pm~2$	12 ± 3	18 ± 2	$7~\pm~2$
Follow (F)	$8~\pm~1$	$43~\pm~5$	$8~\pm~2$	36 ± 4	$8~\pm~2$	38 ± 5
Attempted mount (M)	5 ± 1	305 ± 24	5 ± 1	278 ± 19	$7~\pm~1$	290 ± 17
Genital self-grooming (GG)	3 ± 1	325 ± 27	4 ± 1	$303~\pm~24$	3 ± 1	311 ± 20
Sequences (bouts)						
Total number of bouts (TB)	20 ± 4		23 ± 5		18 ± 4	
% (NSB-GS-NSB)*	60 ± 9		61 ± 11		$44~\pm~7$	
% (NSB-GS-F-NSB)*	15 ± 4		17 ± 3		17 ± 4	
% (NSB-GS-F-M-NSB)*	10 ± 3		$13~\pm~3$		22 ± 4	
% (NSB-GS-F-GL-NSB)*	15 ± 3		9 ± 2		17 ± 3	

Table III. Mating and its inclustructure	Table III.	Mating	and its	microstructure
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These aspects of sexual behavior were compared in observations of mice homozygous for the normal Vitamin D receptor gene (WT) and of their littermates that were either heterozygous (HZ) or homozygous (NM) for this null mutation. A series of individual males of a given type was paired with a female mouse in estrus in the actometer test for 10 min. p > 0.05 between groups (U-test) for all measures. *Percentages of sequences are calculated as % of total bouts (TB = 100%). NSB—non-sexual behavior (any male-performed action that is not oriented toward the female, except GG). the regulation of behavior. Our previously published studies (Kalueff et al., 2004a, b, c) were the first reports characterizing behavioral phenotypes of NM. Recently, some additional behavioral anomalies have been reported for these mice (Burne et al., 2004b), emphasizing the growing interest in the use of the VDR genetic ablation as an animal model of human vitamin D-related brain disorders. The results of the present study demonstrate that mice lacking functional VDR display abnormal grooming, including altered sequencing, frequent interrupted and incomplete bouts and altered regional distribution of both spontaneous and artificially induced grooming. In general, these behavioral findings raise several important questions. Why are grooming behaviors so essential to assess? What is the degree of dominance of the behavioral impairment reported here? How can the loss of VDR lead to impaired sequencing of grooming in mice? What can be potential implications of our study?

In general, there are several reasons to analyze grooming phenotypes in detail. First, this ancient innate behavior represents an essential part of rodent waking behavior (Sachs, 1988; Greer & Capecci, 2003). Second, grooming and its sequential patterning are very sensitive to various exogenous and endogenous factors (including stress and psychotropic drugs), and therefore represent a rich source of behavioral and biological information (Spruijt et al., 1992). Finally, various mutant mice often display altered grooming phenotypes (Cromwell et al., 1998; Strazielle & Lalonde, 1998; Kalueff et al., 2004a). Together, this clearly indicates that an in-depth ethological analysis of grooming of both mutant and background mice represents an important part of behavioral phenotyping in neurogenetics research.

Analyzing the behavioral data presented here, we note that impaired behavioral organization of grooming was only seen in NM (Figs. 1, 2), and that grooming patterns were unaltered in both HZ and WT groups (Fig. 3). This is in line with our previously published studies (Kalueff et al., 2004b, c) showing almost identical behavioral phenotypes of HZ and WT mice in a number of anxiety and motor tests. Consistent with this, numerous observations show that HZ mice display similar VDR gene mRNA expression, have no overt anomalies, and phenotypically are indistinguishable from the WT mice (Yoshizawa et al., 1997; Kato et al., 1999). Moreover, all other mutant mice with non-functional VDR (Li et al., 1999; Erben et al., 2002) also reveal similar phenotypes in HZ and WT groups. Firstly, our results reveal the degree of dominance (harmful recessive) observed for behavioral phenotypes of NM. Secondly, given recent data on reduced VDR level in HZ mice (Yoshizawa et al., 1997; Kallay et al., 2001), our behavioral findings raise the possibility that a limited number of functional VDR may be enough to maintain both normal physiological and behavioral phenotypes in mice. Importantly, numerous clinical data on hereditary vitamin D-resistant rickets (associated with various VDR gene mutations) parallel these data, showing normal phenotypes in heterozygous carriers (Malloy et al., 1997). Taken together, these findings support the notion that that physiological and behavioral anomalies induced by VDR impairment are inherited as a recessive in both mice and humans.

Explaining how the lack of VDR in mice can lead to the behavioral alterations described here, we first suggested that genetic ablation of VDR may affect the brain neurophysiological pathways which control normal grooming sequencing (Aldridge & Berridge, 1998; Berridge, 1998). For example, via VDR vitamin D is known to modulate brain neurotransmitters, including acetylcholine and dopamine (Garcion et al., 2003; Carswell, 1997), both known to regulate grooming sequencing (Spruijt et al., 1992). Given our findings in mutant mice, genetic ablation of VDR in the brain, especially in the structures involved in grooming motor control, may disrupt brain vitamin D-VDR signalling pathways which, associated with disturbed modulation of neurotransmitters in these regions, may cause the abnormal grooming reported in our experiments. Moreover, genetic ablation of VDR, known to affect brain development (Eyles et al., 2003), may alter the formation of certain brain areas (such as basal ganglia and brain stem) involved in the control and regulation of grooming and its sequencing (Berntson et al., 1988; Berridge, 1989). For example, the basal ganglia play a particularly important role in grooming, implementing its sequence by modulating the activation of other competing circuits, such as sensorimotor guided systems (Aldridge & Berridge, 1998; Greer & Capecci, 2002). Interestingly, lesions to other forebrain motor systems, including motor cortex, neocortex or cerebellum, produce sensori-motor deficits in grooming but do not disrupt its patterning (Aldridge & Berridge, 1998). This implies that the basal ganglia control sequencing of grooming and other innate serial behaviors, and that their possible impairment in NM may account for the abnormal grooming patterning seen in the present study.

However, as can be seen in Table III, a detailed ethological analysis of non-grooming mating behavior shows that its sequencing was unaltered in all three groups. This suggests that sequencing of some other complex, non-grooming behaviors may be unimpaired in NM. Indeed, eating and maternal behaviors also appeared to have unimpaired sequencing in these mice (homecage observations). In line with this, no overt stereotypic locomotion (typical for the basal ganglia dysfunctions in rodents; see Fedrowitz et al., 2003 for details) was reported for NM subjected to several behavioral tests such as the open filed and holeboard test (Burne et al., 2004b; Kalueff et al., 2004b, c).

Together, rather than supporting general non-specific sequencing impairments due to the basal ganglia defects in these mice, our findings seem to support the idea that grooming is a complex behavior controlled by multiple brain areas, including the limbic system (Greer & Capecci, 2003). For example, hypothalamus and amygdala, the limbic areas involved in mediation of stress, may also be particularly important for the regulation of grooming and its behavioral organisation (Van Erp et al., 1994; Kruk et al., 1998; Kalueff, 2002). Interestingly, high concentrations of VDR have been found in the limbic system (Walbert et al., 2001), and impaired amygdalar function has been recently suggested for NM (Burne et al., 2004b). Given the important role of these structures in both anxiety and grooming, this possibility is in line with our recent findings showing high anxiety phenotype in NM (Kalueff et al., 2004b) and the results of other groups showing robust stress-evoked disorganization of grooming patterning in rodents (Komorowska & Pellis, 2004). Thus, since increased anxiety per se may produce alterations in rodent grooming, affecting both its activity measures and sequencing (Kalueff & Tuohimaa, 2004), it is possible to speculate that the abnormal grooming sequencing reported in the present study may be associated with altered anxiety levels in these mice (Kalueff et al., 2004b).

Since behavioral effects of genetic mutations may be affected by behavioral features of the animals' parental strains (Crawley, 1999), it was important to consider the role of genetic background in our experiments. The 129 mouse strain, used as genetic background in this study, generally displays callosal dysgenesis, hypoactivity and high anxiety (Crawley, 1999; Rodgers et al., 2002; Wahlsten et al., 2003), also see the Mouse Phenome Database (http://www.jax.org). In addition, some of its sub-strains (including 129/S1) have been reported to show generally low grooming activity (Rodgers et al., 2002; Hossain et al., 2004). We hypothesise that background and mutation-specific phenotypes may overlap, indirectly affecting grooming in the NM. For example,

the anxious phenotype of the 129 strain and the "anxiogenic" VDR null mutation (Kalueff et al., 2004b) may cause additive effects on grooming, leading to its abnormal patterning reported here. For example, this may explain frequent short, incomplete and interrupted bouts of mainly rostral ("anxiety-like") grooming seen in both genotypes in the present study. However, it is also possible that the low-grooming background phenotype interacts with the high-grooming phenotype of NM mice (Kalueff et al., 2004a), collectively disorganizing their grooming patterning. Clearly, further studies are necessary to examine these possibilities in detail. For example, it may be necessary to further assess the strain differences in grooming activity of these mutant mice by comparing grooming and anxiety phenotypes of NM generated on different genetic backgrounds (see Crawley & Paylor, 1997, for details). However, marked differences in both the amount of grooming (Kalueff et al., 2004a) and its behavioral organization (Figs. 1, 2, Table II) between the background and NM animals strongly support the crucial role of VDR genetic ablation in the abnormal grooming patterns reported here.

There have been several additional factors that have to be considered here. For example, it was possible to assume that abnormal neurophysiological processes associated with VDR genetic ablation may result in major sensory disturbances in NM, such as impaired vision, olfaction and/or vestibular system. However, our recent studies have shown that NM have unimpaired olfactory, visual and vestibular systems (Kalueff et al., 2004b, c). Likewise, the fact that all genotypes used here were produced by heterozygous crosses, allowed us to rule out any postnatal influences (e.g., differing maternal styles) from causing contrasting grooming phenotypes in this study. Since NM have been reported to yield severely aberrant skeletal muscle development and rickets-like bone deficits (Endo et al., 2003; Yoshizawa et al., 1997), it was also possible to assume that these abnormalities could impair motor coordination and activity, thus affecting the mouse grooming microstructure. However, the motor dysfunctions reported for NM can only be seen if strong psychophysical stressors are applied, and overt motor/coordination difficulties are not seen in NM subjected to less rigorous tests (Kalueff et al., 2004b, c). Finally, increased (uninhibited) grooming phenotype of these mice (Kalueff et al., 2004a) also negates this "motor deficit" hypothesis.

Another possible factor may be peripheral (cutaneous) effects of VDR genetic ablation, evoking different patterns of grooming activity.

However, the significant portion of multiple-area grooming seen in our study, with a clear cephalocaudal trend in both genotypes, is consistent with centrally-controlled, programmed grooming, rather than stimulusdriven grooming (Table II). Indeed, if altered grooming in NM was merely a response to peripheral stimuli, one would not expect grooming patterns to sequence from one area to another (Ekstein & Hart, 2000). A clear displacement (stress-evoked) character of grooming observed in the NM tested on the horizontal rod (Kalueff et al., 2004a) also negates the role of peripheral factors in abnormal grooming patterning observed in the present study.

Moreover, since vitamin D plays an important role in the regulation of Ca^{2+} homeostasis (Garcion et al., 2003), another possible explanation for our findings in NM may be disregulation of Ca^{2+} homeostasis, usually associated with vitamin D/VDR-related disorders and various behavioral abnormalities in both animals and humans (Altemus et al., 1987; Carswell, 1997). However, the apparent lack of any motor/ coordination defect in these mice tested in mild tests (Kalueff et al., 2004b, c) and the increased (not inhibited) grooming activity in these mice cannot be explained by Ca^{2+} -induced motor defects. Also, no impairment of grooming was reported in the holeboard test in severely hypocalcemic young NM on a mixed C57BL/6-129Sv genetic background (Burne et al., 2004b). Taken together, these findings suggest that Ca^{2+} disregulation per se may not be responsible for the abnormal grooming sequencing in this study.

Can the phenomenon reported here be a behavioral stereotypy? Indeed, it has been suggested that stereotypies may originate from the animals' natural behavioral repertoire or displacement activity (Wurbel et al., 1998). Although grooming fits both possibilities, we note that stereotypies are repetitive invariant patterns associated with decreased flexibility of the stereotyped sequence (Wurbel & Stauffacher, 1998). In contrast, our current results show increased behavioral flexibility of NM grooming sequencing, thus suggesting that impaired patterns of grooming in these mice do not represent stereotypic behavior.

In many species, the genetics of complex patterned behaviors provide many examples in which mutations might lead to excessive but functionally impaired behaviors. In fruit flies, the *dissatisfaction* mutation leads to vigorous courtship but impairs copulation (Finley et al., 1998); the *fru*¹ genotype of the *fruitless* locus impairs copulation without disrupting courtship activity (Villella et al., 1997). A similar situations is

commonly seen in mice. For example, a *Hoxb8* mutation leads to higher grooming activity scores but shows abnormal regional distribution of grooming (Greer & Capecci, 2002), while the *weaver* mutation (affecting both striatum and cerebellum) leads to a greater number of grooming bouts but produces smaller forelimb strokes and incomplete stereotypic sequences (Coscia & Fentress, 1993; Bolivar et al., 1996). To some extent, our present results coincide with these observations, allowing us to speculate that increased grooming activity in NM may be used to "compensate" their mutation-induced impaired grooming sequencing reported here.

In summary, the main conclusion of our study is that mice with ablated VDR display abnormal sequencing of grooming, including increased percentages of incorrect patterns, and transitions (contrary to the cephalocaudal progression). In addition, these mice demonstrate more interrupted and incomplete bouts, as well as the atypical regional distribution of their grooming activity. These phenomena, consistent with a general stress-evoked disruption of grooming sequencing in rodents, may shed light on the endogenous mechanisms that are modulated by the vitamin D/VDR system, and control complex behaviors such as grooming.

What can be potential implications of these results? A growing number of genetic anomalies have been found in the human VDR gene, including missence and nonsence mutations, splice site mutations, and partial deletion of the VDR gene (Malloy et al., 1997, 2002). Such mutations, affecting DNA-binding, nuclear localization, ligand-binding, heterodimerization and other functions of the VDR, lead to partial or total hormone resistance and rickets (Malloy et al., 2002), with many behavioral anomalies (Carswell et al., 1997) sometimes associated with more severe psychiatric phenotypes (Ozer et al., 2004). For the development of effective treatments for such disorders, it is therefore necessary to increase our knowledge on the central function of the vitamin D/VDR system. Analysis of various behaviors (including grooming) in mutant mice with impaired VDR can be a useful tool for such studies, focusing on motor/sequencing and emotional deficits associated with vitamin D/VDR-related disorders.

In general, our data give further support to the crucial role of vitamin D in the brain (Carswell, 1997; Garcion et al., 2002) and contribute to the growing recognition of the importance of VDR in the regulation of behavior (Burne et al., 2004b; Kalueff et al., 2004a, b, c).

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