ELSEVIER

Physiology & Behavior 82 (2004) 405–409

PHYSIOLOGY & BEHAVIOR

Increased grooming behavior in mice lacking vitamin D receptors

A.V. Kalueff[a,*], Y.-R. Lou[a,1], I. Laaksi[b,1], P. Tuohimaa[a,c,2]

[a]Department of Anatomy, Medical School, Tampere University, Tampere 33014, Finland
[b]Department of Cell Biology, Medical School, Tampere University, Tampere 33014, Finland
[c]Department of Clinical Chemistry, Tampere University Hospital, Tampere 33014, Finland

Received 29 September 2003; received in revised form 8 April 2004; accepted 15 April 2004

Abstract

Vitamin D is a neuroactive secosteroid with several important functions in the nervous system. Many human and animal findings link alterations in the vitamin D system to various neurological and behavioral disorders. Since grooming is an important element of animal behavior, here we studied whether genetic ablation of vitamin D receptors (VDR) in mice may be associated with altered grooming behaviors. Overall, VDR knockout (VDRko) mice presented longer duration and higher frequency of grooming when tested in the actimeter, open field, elevated plus maze, and horizontal rod tests. Increased grooming did not, however, correlate with unaltered general activity level (actimeter test), anxiety-like behaviors (hole board and elevated plus maze tests), and emotional reactivity index (defecation boli). In general, our results confirm the role of vitamin D and VDR in the regulation of behavior, including grooming, and suggest that increased grooming behavioral phenotype may be associated with genetic ablation of VDR in mutant mice.

Keywords: Vitamin D receptors; Knockout mice; Grooming behavior

1. Introduction

Neurosteroid vitamin D plays an important role in the nervous system including differentiation, regulation of Ca^{++} homeostasis, modulation of neurotrophins release, and activity of key brain genes and enzymes of neurotransmitter metabolism [3,6]. The hormonal functions of vitamin D are mediated through the vitamin D receptor (VDR), a member of the nuclear receptors superfamily of ligand-activated transcriptor factors [6]. VDR are widespread in the brain and the spinal cord, including the areas involved in regulation of motor activity and behavior [10,11,14]. Several clinical and experimental studies outline the possible role of vitamin D and VDR in the regulation of various behaviors [1,3]. Self-grooming is an important part of animal behavioral repertoire [9,16], which in rodents has long been considered as a complex ethologically "rich" response, particularly sensitive to various endo- or exogenous factors [7,12,13]. Grooming normally proceeds in a cephalocaudal direction and consists of licking the paws, washing movements over the head, fur licking, and tail/genitals cleaning [7,9,13]. Recently, high concentrations of VDR were found in hypothalamus, the limbic system, and basal ganglia [10,11,14], the brain areas known to be involved in neural control of grooming [9,13,16]. These data suggest that VDR may be important for the regulation of grooming behaviors, thus requiring further experimental investigation.

Knockout animals provide a powerful tool extensively used in the field of neurobehavioral research [4,5]. Mice with genetically ablated VDR are currently available for biomedical research [17] to investigate the role of vitamin D and VDR in various physiological processes. Our preliminary observations in the home cage showed that VDR knockout (VDRko) mice appear to have similar motor activity levels but usually tend to spend more time grooming than do the wild-type (WT) animals. Although grooming in rodents plays a very important role [7,9], in many studies this behavior has merited little scrutiny, frequently addressed only cursorily among other measures (for details, see Ref. [7]). As such, in the present study in VDRko mice, we wanted to focus exclusively on the assessment of possible variation in their grooming behaviors. Here we

* Corresponding author. Tel.: +358-3-2156640; fax: +358-3-2156170.
E-mail address: avkalueff@inbox.ru (A.V. Kalueff).
[1] Tel.: +358-3-2156640; fax: +358-3-2156170.
[2] Tel.: +358-3-2156726; fax: +358-3-2156170.

0031-9384/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.physbeh.2004.04.010

characterize in detail grooming activity of VDRko mice and assess the potential role of VDR in grooming by comparing grooming patterns of VDRko and the WT animals tested in a battery of behavioral tests.

2. Materials and methods

Adult male mice aged 24–30 weeks were maintained in a virus/parasite-free facility under conditions of controlled temperature (22 ± 2 °C), humidity (60%), and a 12:12-h light–dark cycle (lights on 7:00 a.m.). VDR gene mutant mice were bred in the University of Tampere, Finland, from the line initially generated in the University of Tokyo, Japan [17]. In the present study, 12 homozygous (−/−) VDRko mice were compared to 12 homozygous (+/+) WT 129/S1 mice. Mice of both genotypes were littermates produced by heterozygous crosses for four generations. The animals were housed in groups of four in standard laboratory cages (20 × 34 × 16 cm). Water and food were available ad libitum (except food-finding experiments, when in order to increase hunger, animals were food deprived for 10 h prior to testing). In the present study, the animals were tested in a battery of behavioral tests including actimeter, open field, hole board, elevated plus maze, and horizontal rod tests. All testing was conducted between 1700 and 1900 h. During the testing sessions, the experimenter remained standing in front of (and 2 m away from) the testing boxes. Three ethological measures of grooming activity were evaluated in all these tests: (1) frequency (the number of grooming bouts), (2) total time (s) spent grooming, and (3) average duration of a single grooming bout (s) calculated as total time spent grooming divided by the number of bouts. In addition, defecation index (the number of boli deposited) was scored as the conventional emotionality index in each test. The actimeter was a plastic box (30 × 30 × 30 cm) with a floor divided into four squares (15 × 15 cm). On the day of the experiments, each mouse was placed separately in the actimeter where its behavior was recorded for 5 min. In addition to measuring grooming and defecation, in this test we assessed animal general motor activity—horizontal (the number of squares visited, four paws) and vertical activity (the number of times an animal stood erect on its hind legs with forelegs in the air of against the wall). This experiment was the first exposure of the mice to the behavioral testing. One month later, grooming behavior was assessed for 5 min in the open field, and 14 days later, hole board tests. The open field and hole board tests were a plastic box (45 × 45 × 45 cm) with a floor divided into nine squares (15 × 15 cm). The hole board removable floor had 64 holes (1.5 cm in diameter). The behavioral measures in this test also included horizontal and vertical activity and the number of holes inspected by nose poking. Fourteen days later, the animals were exposed to the elevated plus maze for 5 min. This test consisted of a Plexiglas cross-shaped maze, 50 cm high, with two opposing open arms (50 × 10 cm), and two opposing closed arms (50 × 10 × 15 cm) interconnected by a central platform (10 × 10 cm). The behavioral measures in this test also included the number of open arm entries and the number of closed arm entries (four-paw criterion). In addition, several ethologically derived indices were calculated based on these parameters, including total number of arm entries (open + closed), the ratio of open–closed arm entries, and the ratio of open–total arm entries. Fourteen days later, the mice were tested in the horizontal rod test. The test was a horizontal metal rod (30 cm long, 1.5 cm in diameter), fixed to a platform elevated 30 cm from the floor. The animals were placed individually to the center of the horizontal bar, and their grooming activity was assessed for 3 min. Ten days later, the olfactory abilities of the mice were analyzed in a food-finding test following a 10-h food deprivation time. The animals were placed in a plastic box (50 × 50 × 50 cm) and after a 5-min acclimation time, the food (1 × 1 × 1 cm cheese cube) was introduced in the diagonally opposite corner of the box. The latency (s) of finding food, the number, and the duration (s) of contacts with food (sniffing, licking, biting, and eating) for 3 min were used as a measure of animal olfactory system. Between subjects, each apparatus was thoroughly cleaned (wet and dry cloths). All animal experiments were performed in full compliance with the Finnish laws on animal experimentation and approved by the Ethical Committee of the University of Tampere.

2.1. Statistical analysis

All data are presented as mean ± S.E.M. Data were analyzed by Mann–Whitney two-tailed *U* test. In all tests, a probability of less than .05 was considered statistically significant.

3. Results

In the actimeter test, the WT and VDRko mice demonstrated similar levels of horizontal (41 ± 6 and 37 ± 7, respectively, $P>.05$) and vertical activity (29 ± 5 and 25 ± 5, respectively, $P>.05$). The only marked behavioral difference found in this test, as can be seen in Table 1, was the increase in grooming activity (higher frequency, longer total time spent grooming, and a trend towards longer average duration of a single bout) in the VDRko mice compared to the WT mice. In the open field test, VDRko mice again groomed more and longer than did their WT counterparts, also showing significantly longer average duration of a single bout (Table 1). In the hole board test, the VDRko animals showed a similar tendency for increased grooming activity (although this was not statistically significant; Table 1), also demonstrating less horizontal activity and unaltered vertical activity and holes exploration (Fig. 1). In the elevated plus maze test, a significant increase in the VDRko group was found for the total time spent grooming

Table 1
Grooming of the WT and VDRko mice tested in a battery of behavioral tests

Tests	Number of grooming bouts		Total time spent grooming (s)		Average duration of a single bout (s)		Number of defecation boli	
	WT	VDRko	WT	VDRko	WT	VDRko	WT	VDRko
AM	2.0 ± 0.4	3.7 ± 0.8 *	8.3 ± 1.5	22.3 ± 4.7 *	4.2 ± 0.8	6.0 ± 1.1	0.8 ± 0.3	1.1 ± 0.4
OF	0.9 ± 0.3	1.3 ± 0.3	4.6 ± 1.0	11.9 ± 2.4 *	5.1 ± 0.9	9.2 ± 1.3 *	1.0 ± 0.5	1.4 ± 0.6
HB	0.9 ± 0.3	1.2 ± 0.4	3.2 ± 1.2	6.6 ± 1.5	3.6 ± 1.4	5.5 ± 1.6	0.9 ± 0.4	1.2 ± 0.6
EPM	1.4 ± 0.3	1.2 ± 0.3	8.5 ± 2.1	14.9 ± 2.1 *	6.1 ± 1.3	12.4 ± 2.1 *	0.8 ± 0.3	0.9 ± 0.3
HR	0	1.4 ± 0.3 * *	0	4.5 ± 0.9 * *	0	3.2 ± 0.4 * *	1.2 ± 0.3	1.4 ± 0.4

AM—actimeter, OF—open field, HB—hole board, EPM—elevated plus maze, HR—horizontal rod tests. All data are presented as means ± S.E.M.
* $P<.05$, compared to the WT group (U test).
** $P<.01$, compared to the WT group (U test).

and the duration of a single grooming bout (Table 1). However, we found no difference in any other behavioral parameter: the number of open, closed, and total arm entries, as well as the ratio of open–closed or open–total arm entries (Fig. 1). When placed on the horizontal rod, the VDRko mice demonstrated grooming activity, which in this test was completely lacking in the WT group (Table 1). In addition, no marked difference was observed for defecation scores (the number of defecation boli) in any of the tests (Table 1). Finally, the WT and VDRko mice displayed similar olfactory abilities, as assessed in the food-finding test (the latency of finding food: 15 ± 3 and 15 ± 4 s, respectively, $P>.05$; the number of contacts with food: 9 ± 2 and 10 ± 1, respectively, $P>.05$; total duration of contacts with food: 36 ± 7 and 42 ± 6 s, respectively, $P>.05$).

4. Discussion

The results presented here show the contrasting differences in grooming behavior in mice lacking VDR compared to WT animals. To our knowledge, this is the first ethological study of VDRko mice focusing on their grooming behaviors assessed in a set of well-validated experimental paradigms. Overall, the results indicate that during the tests, the VDRko mice generally displayed more grooming compared with the WT mice. This increase of grooming activity in VDRko mice was seen consistently in all tests used in the present study and was statistically significant (except for hole board test, where 1.5- to 2-fold increase in grooming did not reach significance). Together, this raises the possibility that increased grooming in VDRko mice may be a specific behavioral phenotype possibly attributable to disturbed vitamin D-VDR system in these animals.

How specific is this behavioral abnormality in VDRko mice? Analyzing our data, we first considered the possibility that the activation of grooming reported here may be due to possible difference in general motor activity levels between VDRko and WT mice. However, in the present study, we found that the VDRko mice did not show any difference in motor activity levels in the actimeter test. In line with this, behavioral measures reflecting the motor activity component in the elevated plus maze, such as closed arm and total arm entries [12,15], were unaltered in both groups (Fig. 1). Together, this allowed us to discount the "motor activity" factor while interpreting the results of our study.

Other potentially important factor to be considered in the present study was the possibility that increased grooming in the VDRko mice may be due to alterations in their emotional behaviors, especially anxiety, known to affect animal performance in all these tests [4,12]. Indeed, since grooming, especially in rodents, is particularly responsive to the level of stress [7,13], it was possible to assume that the increased grooming activity in our VDRko mice might be

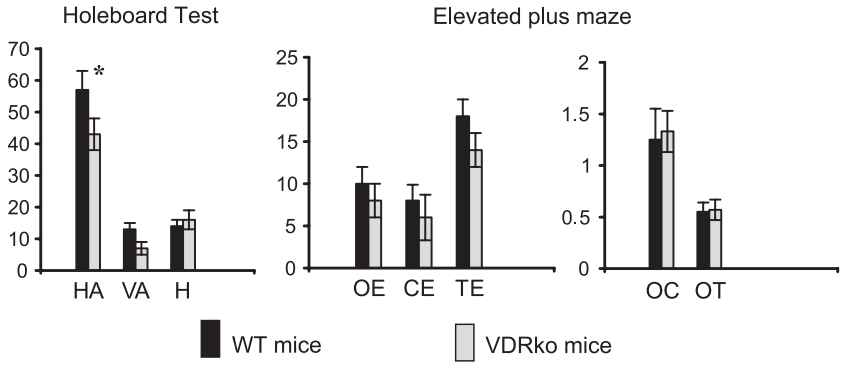

Fig. 1. Assessment of anxiety level in VDRko compared to WT mice tested in the hole board and elevated plus maze tests. HA—horizontal activity, VA—vertical activity, H—holes inspected, CE—closed arm entries, OE—open arm entries, TE—total arm entries, OC—ratio of open–closed arm entries, OT—ratio of open–total arm entries. * $P<.05$, compared to the WT group (U test).

due to increased stress or anxiety. Since the degree of stress varies in the different test situations used in our study, from low-stress actimeter to high-stress elevated plus maze and horizontal rod tests [4,5,15], we were interested whether grooming increase might vary in different tests. However, in our experiments, essentially the same two- to threefold increase of grooming occurred in all these tests (Table 1). This indicates that increase in grooming in the VDRko mice did not correlate with the stressfulness of the tests, thus negating the "emotionality" hypothesis of increased grooming phenotype in the these mice. Consistent with this, the VDRko mice demonstrated no clear link between increased grooming and anxiety in the elevated plus maze (Fig. 1), one of the most sensitive animal models of anxiety [12]. Indeed, in this test increased grooming was observed in the VDRko mice, while the traditional anxiety indices, such as the number of open arm entries, the ratio of open–total and open–closed arm entries, [12,15] remained unaltered. A similar profile was seen in the hole board test, another popular anxiety model [4]. Here the VDRko mice demonstrated a tendency to increased grooming (Table 1) together with unaltered anxiety measures (the number of holes explored and vertical activity) (Fig. 1) and some decrease of horizontal activity, which might be attributed to simultaneous increase in grooming. Moreover, in all tests used in the present study, grooming activation did not correlate with the defecation scores (Table 1), traditionally used as an emotional reactivity index [4,5]. Although defecation alone may serve as a vague index of emotionality, especially when low average number of boli is emitted, the latter several consistent observations collectively do not seem to support the role of emotionality in our results. Although the possible differences in emotional behaviors between the VDRko and the WT mice were not the object of in-depth analysis in this study, and may indeed need to be studied further in the future, our data suggest that increased grooming in VDRko mice is not related to changes in their level of stress or emotionality. Furthermore, stress has long been known to synchronize grooming behavior by decreasing its individual variability [13]. However, analysis of S.E.M. data (Table 1) shows that in all tests used here the VDRko mice displayed equal or even higher variability of grooming measures compared to the WT group. Finally, rodent grooming behaviors elicited by various stressors are characterized by frequent and abbreviated grooming bouts [7,16]. However, analysis of the average duration of grooming bouts of the mice tested in our study (Table 1) clearly shows that shortening of bout duration did not occur in our experiments. Quite the reverse, a general and consistent trend towards longer grooming bouts in the VDRko group was seen in all behavioral tests, particularly in the open field, elevated plus maze, and horizontal rod tests, where this effect was statistically significant. Together, these observations give additional support to the conclusion that activated grooming in VDRko mice is not their behavioral response to stress or anxiety.

In general, our data show that increased grooming activity in VDRko mice can be dissociable from changes in animals' general activity and/or emotionality. However, another potential explanation for our data could be that abnormal neurophysiological processes associated with VDR genetic ablation may result in major sensory disturbances in VDRko mice, such as impaired vision, olfaction, and/or vestibular system. Disturbances in these systems are known to lead to particularly marked abnormalities in grooming behavior [7], and this possibility is therefore to be examined in detail. In the present study, we demonstrated that VDRko mice have unimpaired olfactory system, as assessed in the food-finding test. Recently, we have also shown that these mice appear to have unimpaired visual and vestibular systems, as assessed in the novel object-finding and the horizontal rod balancing tests [8]. Collectively, these findings allowed us to rule any possible role of sensory disturbances in the increased grooming activity demonstrated by the VDRko mice in the present study.

Taken together, it is therefore possible to suggest that increased grooming phenotype may be a specific behavioral feature of mice lacking VDR gene. How can the loss of VDR lead to the phenomenon described in our study? First, we can suggest that genetic ablation of VDR may affect the brain neurophysiological mechanisms and pathways that control normal grooming [9,13]. For example, vitamin D has been reported to be involved in VDR-mediated modulation of brain neurotransmitters, including acetylcholine and dopamine [3,6], known to regulate grooming [7]. It is therefore possible to suggest that imbalance of these neuromediators in VDRko mice may result in disturbed behaviors such as the increased grooming seen in the present study. Alternatively, genetic ablation of VDR, known to affect brain development [3,4], may alter the formation of certain brain areas (such as hypothalamus, limbic system, and basal ganglia) involved in the control and regulation of grooming behavior. The latter may again result in abnormal patterns of regulation of grooming seen in VDRko animals. Thirdly, one has to consider the important role of VDR in the regulation of Ca^{++} [6] and the crucial role of Ca^{++} for motor functions [1,3]. A causal link between disturbances in Ca^{++} and various behavioral abnormalities in both animals and humans has been reported in the literature [1,3]. Since VDR mediate vitamin D regulation of plasma and brain Ca^{++} [3,6], behavioral abnormalities such as the increased grooming in VDRko mice seen in our experiments may be attributed to the disturbed Ca^{++} homeostasis previously reported for these mice [8,17].

In addition, since vitamin D is synthesized in the skin after sun exposure and was found in body grease and fur [2,6], certain animal behaviors, such as outdoor activities and self-grooming, may represent important physiological adaptations of the organism in respect to regulation of the vitamin D system. For example, the evolutionary significant link between vitamin D and grooming behavior [2] might lead to a very interesting hypothesis, requiring further

testing, as to why abnormal grooming might be observed in animals with disturbed vitamin D/VDR system.

Lastly, it is known that several behavioral disorders in humans and animals are associated with vitamin D dysfunctions [1,3]. Stereotypic behaviors are repetitive inappropriate responses and represent a common symptom of many behavioral disorders [3]. Since grooming is a common stereotypy in both animals and humans [7], it therefore seems possible that increased grooming activity may be some kind of behavioral stereotypy, typical for VDRko mice. Consistent with this, in all tests, prolonged time spent grooming was the most marked behavioral alteration in the VDRko group, which also demonstrated 1.5- to 2-fold increase in the average duration of a single grooming bout (Table 1). Moreover, in the horizontal rod test, where retaining their balance on the rod was crucial for mice, grooming was not seen in the WT mice. However, as can be seen in Table 1, VDRko mice did demonstrate this "inappropriate" behavior while tested in this paradigm. Taken together, this lends indirect support to the hypothesis that in VDRko mice increased grooming might represent stereotypic behavior. This contention will certainly require further experimentation to be confirmed. For example, it would be of interest to explore if the behavioral characteristics of animals with vitamin D deficiency and/or impaired VDR might include some other (i.e., nongrooming) stereotypic behaviors. However, if true, this might explain the stereotypies frequently seen in patients with vitamin D-related disorders [3] and, based on VDR ablation in mice, could lead to the development of several interesting animal models of vitamin D-related stereotypic behavioral disorders.

In summary, the main conclusion of our study is that mice with ablated VDR receptors display increased grooming behavioral phenotype. Since increased grooming behavior may not be the only behavioral peculiarity of VDRko mice, a detailed behavioral analysis (especially focusing on behaviors that are ethologically strongly related to grooming, for example, parental, sexual, and aggressive behaviors) seems to justify further studies of these mutant mice. In general, our data give support to the long-standing and continuously mounting evidence for the role of vitamin D in the brain [3,6,11] and contribute to the growing recognition of the importance of VDR in the regulation of behaviors. Clearly, more work is needed to understand the role of the vitamin D/VDR system in the regulation of various normal and pathological behaviors in animals and humans.

Acknowledgements

This research was supported by the grants from CIMO, Tampere University Hospital, and the Academy of Finland. The authors are greatly indebted to Prof. S. Kato (University of Tokyo, Japan) for providing the initial knockout mice for our research.

References

[1] Altemus KL, Finger S, Wolf C, Birge SJ. Behavioral correlates of vitamin D deficiency. Physiol Behav 1987;39:435–40.
[2] Carpenter KJ, Zhao L. Forgotten mysteries in early history of vitamin D. J Nutr 1999;129:923–7.
[3] Carswell S. Vitamin D in the nervous system: actions and therapeutic potential. In: Feldman D, Glorieux FH, Pike JW, editors. Vitamin D. San Diego: Academic Press; 1997. p. 1197–211.
[4] Crawley J. Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. Brain Res 1999; 835:18–26.
[5] Flint J, Corley R, DeFries JC, Fulker W, Gray JA, Miller S, et al. A simple genetic basis for a complex psychological trait in laboratory mice. Science 1995;268:1432–5.
[6] Garcion E, Wion-Barbot N, Montero-Menei C, Berget F, Wion D. New clues about vitamin D functions in the nervous system. Trends Endocrinol Metab 2002;13:100–5.
[7] Kalueff AV. Grooming and stress. Kiv: KSF Publishers; 2002.
[8] Kalueff AV, Lou Y-R, Laaksi I, Tuohimaa P. Impaired motor performance in mice lacking neurosteroid vitamin D receptors. Brain Res Bull 2004 [in press].
[9] Kruk MR, Westphal KGC, VanErp AMM, VanAsperen J, Cave BJ, Slater E, et al. The hypothalamus: cross-roads of endocrine and behavioral regulation of grooming and aggression. Neurosci Biobehav Rev 1998;23:163–77.
[10] Langub MC, Herman JP, Malluche HH, Koszewski NJ. Evidence of functional vitamin D receptors in rat hippocampus. Neuroscience 2001;104:49–56.
[11] Prufer K, Veenstra TD, Jirikowski GF, Kumar R. Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the rat brain and spinal cord. J Chem Immunol 1999;16:135–45.
[12] Rodgers RJ, Cole JC. The elevated plus-maze: pharmacology, methodology and ethology. In: Cooper SJ, Hendrie CA, editors. Ethology and psychopharmacology. Chichester: Wiley; 1994. p. 9–44.
[13] vanErp AMM, Kruk MR, Meelis W, Willekens-Bramer DC. Effect of environmental stressors on time course, variability and form of self-grooming in the rat: handling, social contact, defeat, novelty, restraint and fur moistening. Behav Brain Res 1994;65:47–55.
[14] Walbert T, Jirikowski GF, Prufer K. Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the limbic system. Horm Metab Res 2001;33:525–31.
[15] Wall PM, Messier C. Methodological and conceptual issues in the use of the elevated plus-maze as a psychological measurement instrument of animal anxiety-like behavior. Neurosci Biobehav Rev 2001;25: 275–86.
[16] Whishaw IQ, Nonneman AJ, Kolb B. Environmental constrains on motor abilities used in grooming, swimming, and eating by decorticate rats. J Comp Physiol Psychol 1981;95:792–804.
[17] Yoshizava T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, et al. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat Genet 1997;16:391–6.