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Impaired motor performance in mice lacking neurosteroid vitamin D receptors

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Abstract

Vitamin D is a neuroactive seco-steroid and its importance to the nervous system is receiving increasing recognition. Since numerous data link vitamin D dysfunctions to various neurological and behavioural disorders, we studied whether genetic ablation of vitamin D receptors (VDR) may be associated with motor impairments in mice subjected to several behavioural tests. The data obtained in the vertical screen and swim tests show that VDR genetic ablation produces severe motor impairment (shorter screen retention and poor swimming) in mutant mice compared to wild-type and heterozygous control animals. These impairments appear to be unrelated to visual, vestibular and activity/emotionality parameters of mice, and are likely associated with disturbed calcium homeostasis. This study confirms the important role of the vitamin D system in motor functions and suggests that animal genetic models targeting the vitamin D/VDR system may be a useful tool to study vitamin D-related motor/behavioural disorders.

Keywords: Vitamin D receptors; Knockout mice; Motor performance; Swimming behaviour

1. Introduction

Neurosteroid hormone vitamin D plays an important role in the nervous system including differentiation, regulation of Ca^{2+} homeostasis, modulation of neurotrophins release and activity of key brain genes and enzymes of neurotransmitter metabolism [2,7]. The functions of vitamin D are mediated through the nuclear vitamin D receptor (VDR) [7,26]. VDR gene expression has been demonstrated in neurons and glial cells [7]. VDR are widespread in the brain and the spinal cord including the areas involved in the regulation of motor activity and behaviour [11,17]. Several studies outline the possible role of vitamin D and VDR in regulation of motor behaviour. In humans, vitamin D deficiency is often accompanied by tetany, seizures and other motor disorders [2]. In animals, vitamin D deficit produces behavioural alterations including decreased exploration and maze performance [1]. Together, this evidences that vitamin D system may be an important factor controlling motor functions in animals and humans. Various VDR disorders may therefore be a risk factor for abnormal locomotion in both humans and animals – an area of research, which is becoming increasingly important. Since motor abnormalities have been reported for a number of mutant mice [5,6,22], transgenic and knockout mice represent an increasingly popular tool to study the role of gene functions in motor behaviour. To examine the link between vitamin D-related disorders and motor activity, we studied VDR knockout mice (VDR-KO) currently available for biomedical research [14,26]. The importance of assessing the status of locomotory system is now widely recognised as a key part of behavioural phenotyping of mutant animals [3,4]. Because, to the best of our knowledge, there are no published data describing motor behavioural profiles of VDR-KO mice, in our research we wanted to assess the motor behaviours of these mutant animals in detail. Here, we present data based on screening of VDR-KO mice in a battery of behavioural tests assessing their general motor activity, visual abilities and motor performance.

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2. Materials and methods

Adult male mice aged 24–30 weeks were maintained in a virus/parasite-free facility and exposed to a 12-h light, 12-h dark cycle (lights on: 07:00 h). VDR-KO mice were bred in the University of Tampere from the line initially generated in the University of Tokyo (Japan) [26]. In the present study, homozygous (−/−) VDR-KO were compared to homozygous (+/+) wild-type 129/S1 and heterozygous (+/−) mice. Mice of all three genotypes were littermates produced by heterozygous crosses. The genotypes of the animals were analysed using the method previously described [10]. To normalize the blood mineral ion levels, VDR-ablated animals were fed a diet containing 2% calcium, 1.25% phosphorum, and 20% lactose supplemented with 2.2 IU vitamin D/g (Lactamin AB, Sweden). All testing was conducted between 17:00 and 19:00 h.

2.1. Motor/coordination tests

In these experiments, ten VDR-KO were compared to ten wild-type and ten heterozygous mice. On test days, the animals were transported to the dimly lit laboratory and left undisturbed for 2 h prior to testing. General locomotor activity was measured for 10 min. in a plastic actometer box (30 cm × 30 cm × 30 cm) with a floor divided into four squares (15 cm × 15 cm). Conventional measures were horizontal activity (the number of squares visited with four paws), vertical activity (the number of times an animal stood erect on its hind legs with fore legs in the air or against the wall) and the number of defecation boli deposited. This experiment was the first exposure of the mice to behavioural testing. Ten days later, the visual sensory abilities and motor coordination of the mice were analysed in a novel object-finding test. The animals were placed in a plastic box (50 cm × 50 cm × 50 cm; with a floor divided into nine squares of 15 cm × 15 cm) and after a 5-min acclimation time, the novel object (5-cm metal sphere) was introduced in the diagonally opposite corner of the box. The latency (s) of finding the sphere was used as a measure of the animals' visual abilities; the number of contacts (the number of times an animal stood on its hind-legs with forelegs placed on the sphere) was used as a measure of animal motor coordination/manipulatory activity. Ten days later, the motor performance of mice was assessed in the vertical screen test as described [22], with some modifications. Briefly, the mouse was placed on the centre of the screen consisting of a plastic frame (30 cm high and 15 cm wide, with 10-cm top and side walls) covered by a plastic net (2-mm mesh) elevated to a height of 60 cm from the floor. The screen was turned immediately to the vertical position with the mouse facing the upper end, and the retention time (the latency to fall off from the screen, s) was measured. To avoid any harm to the animals caused by falling from the screen, a thick cloth was placed under the screen. In addition, emotional reactivity was assessed by the number of defecation boli deposited during the first 5 min of the test. Between subjects, each apparatus was thoroughly cleaned (wet and dry cloths).

2.2. The swim test

Twenty days later, animal motor performance was assessed in the swim test. In this experiment, six VDR-KO mice were compared to five homozygous wild-type and five heterozygous controls. To rule out the impact of phenotypical difference in mice appearance (hairless VDR-KO versus normal haired control groups), the hair of animals from both control groups was removed by shaving. Animals from all groups were anaesthetized with Hypnorm, and the shaving of the control mice was performed using small electric shears. Seven days later, behavioural testing was performed in a water tank consisting of a glass cylinder 50 cm tall and 30 cm in diameter filled with water (25 °C) to a height of 15 cm from the top. The mice were gently put onto the surface of the water, and their swimming behaviour was studied by an experienced investigator for a period of 3 min. The observer recorded the duration and number of immobility episodes (animal remaining motionless in the water with no limb movements except for small postural adjustments or minor head movements), the number of rotations (360° body turns), the number of defecation boli deposited and the number of sinking episodes (animal's nose going below the surface with the body in a vertical position head up). If the mice appeared to be drowning, they were picked up immediately. The water was changed after each animal was tested.

Animal care and experimental procedures were conducted in accordance with the European legislation and the guidelines of the National Institutes of Health. All animal experiments were approved by the Ethical Committee of the University of Tampere. All results are expressed as mean ± S.E.M. Behavioural data were analysed by the Kruskal–Wallis test followed by post-hoc Mann–Whitney test. A probability of less than 0.05 was considered statistically significant.

3. Results

Table 1 summarises data obtained during the assessment of general locomotor activity of VDR-KO and the wild-type and heterozygous control mice. In the actometer test, all three groups demonstrated similar levels of horizontal and vertical activities and the number of defecation boli deposited. Additionally, mice from all these groups displayed similar visual abilities and motor coordination, as assessed in the novel object-finding test (Table 1).

In the vertical screen test, the VDR-KO group demonstrated generally shorter retention time compared to both control groups, while no changes were seen in the number of defecation boli deposited. In the swim test, swimming patterns of the shaven wild-type and heterozygous mice were

Table 1
Assessment of general activity, visual functions and motor performance of VDR knockout mice (VDR-KO) compared to wild-type (WT) and heterozygous (HZ) control animals

Tests/behaviours	Groups			
	WT mice ($n = 10$)	HZ mice ($n = 10$)	VDR-KO mice ($n = 10$)	H values
Actometer test				
Horizontal activity	70 ± 8	71 ± 10	65 ± 11	1.94
Vertical activity	54 ± 8	58 ± 11	50 ± 9	1.53
Number of defecation boli	6 ± 2.0	5 ± 1.7	4 ± 1.5	2.14
Novel object test				
Latency to find the object (s)	17 ± 4	18 ± 4	20 ± 7	2.07
Contacts with the object	5 ± 1.2	5 ± 1.2	4 ± 1.4	1.19
Vertical screen test				
Screen retention time (s)	1457 ± 183	1321 ± 177	647 ± 196	6.61*
Number of defecation boli	4 ± 1.0	5 ± 1.1	3.5 ± 1.4	2.84
Swim test	Shaven WT mice ($n = 6$)	Shaven HZ mice ($n = 6$)	VDR-KO mice ($n = 6$)	
Duration of immobility (s)	28.3 ± 2.7	25.3 ± 1.9	2.3 ± 1.1	13.80***
Number of immobility episodes	5.3 ± 0.9	4.5 ± 1.2	0.4 ± 0.1	12.48**
Number of sinking episodes	0	0	6.0 ± 0.6	16.15****
Number of rotation	0.2 ± 0.05	0.2 ± 0.07	18.0 ± 4.2	10.68**
Number of defecation boli	4 ± 1.1	6 ± 1.7	4 ± 1.6	1.65

Data are presented as mean ± S.E.M. H, the statistic for the Kruskal–Wallis test (d.f. = 2 for all measures). *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$, and ****$P < 0.0005$ – significant difference between genotypes.

similar, including approximately equal number of immobility episodes and total immobility duration (Table 1). In all three groups, no changes were seen in the number of defecation boli. However, we found severe impairment of swimming behaviour in the VDR-KO mice, including swimming in a predominantly vertical body position, very short duration of immobility, frequent stereotypic rotations and specific catatonic-like upper limb spasms (seen most of the time except for short active swimming episodes). Another striking finding, as can be seen in Table 1, was that all the VDR-KO animals demonstrated frequent sinking – an abnormal pattern not seen in the normal swimming of the control animals.

4. Discussion

The vertical screen test is a widely used tool to assess animal motor abilities [19,22]. Using this test, we found a marked reduction of screen retention in the VDR-KO group, indicating severe motor deficits in these mice compared to the wild-type and heterozygous animals. Measurement of mouse behaviours in a tank of water is another popular model for studying rodent behaviour [3,24], including the assessment of motor activity in mutant mice [5,6,12]. Our preliminary observations in this test showed that the VDR-KO mice differ from the wild-type or heterozygous littermates, swimming in a predominantly vertical position, struggling throughout the test, and unable to remain immobile because they will sink as soon as stop moving (data not shown). Although this is in line with the motor deficit we found in the vertical screen test, we noted that VDR-KO mice develop secondary alopecia [14,26] and therefore differ phenotypically from both control groups. Thus, it was possible to assume that hair provides additional buoyancy, raising the possibility that poor swimming in VDR-KO mice may be merely due to the lack of hair. To examine this possibility in the present study, we shaved all the control animals and compared their swimming patterns to those of VDR-KO mice. In line with our earlier observations, shaven mice from both control groups demonstrated normal swimming patterns similar to those of unshaven animals. In contrast, the VDR-KO group demonstrated abnormal swimming, which was markedly different from that of both control groups (Table 1). Overall, despite a dramatic reduction of immobility in an attempt to avoid sinking, these mice still could not perform proper swimming, demonstrating frequent rotation and sinking. This experiment clearly indicates that specific motor impairment in this group is not due to alopecia. Notably, the impaired swimming of the VDR-KO animals also cannot be explained by alteration in the physical properties of the body (weight or density) reported for VDR-KO mice developing 40% decrease in bone density [26]. Clearly, such changes in bone mineral density should have assisted, not impended, animal swimming. Thus, the poor swimming performance of the VDR-KO mice, despite the obvious advantage of less bone density, accentuates the degree of impaired motor functions likely associated with VDR genetic ablation.

However, abnormal swimming behaviour, including inability to swim and repetitive circling, can also be attributed to vestibular dysfunctions [9,13]. Since these patterns were seen in the VDR-KO group, an impairment of the vestibular system has also been considered to explain these results. To address this problem, in our earlier experiments we tested ten

VDR-KO mice on the horizontal bar (a metal bar 1 cm thick, fixed to a platform elevated 30 cm from the floor), similar to the test described in [15]. The mice were placed on the bar and the time for which kept their balance was measured for up to 3 min. Overall, VDR-KO mice demonstrated unaltered horizontal bar retention time compared to both control groups (own unpublished data). These observations are in line with recent data showing that abnormal swimming may occur in mutant lines with unaltered vestibular functions [13], and with the data reported here showing no apparent vestibular-specific motor abnormalities in the VDR-KO mice subjected to the actometer and novel object-finding tests (Table 1). Taken together, this suggests firstly that the vestibular system is unaltered in these animals, and secondly that it cannot be responsible for the abnormal swimming of VDR-KO mice. The same conclusion applies to the visual abilities and navigation of the VDR-KO mice, which are indeed important for animal motor performance in the swim test [12], but appear to be unaltered in VDR-KO mice as assessed in the novel object-finding test (Table 1).

Importantly, in all tests used in the present study, the motor performance of heterozygous mice was similar to that of wild-type mice. These behavioural observations are consistent with earlier data showing that heterozygous mice, possessing 50% of mutated VDR gene copies, show similar VDR gene mRNA expression levels, have no overt abnormalities and phenotypically are indistinguishable from the wild-type animals [14,26]. Another important observation is that no difference was observed in the traditionally used emotionality index (defecation boli) in all behavioural tests used in the present study (Table 1). Furthermore, the actometer test revealed that all three groups demonstrated similar basal levels of locomotory activity, while the assessment of sensory (visual) abilities and vestibular system did not reveal any marked difference in the VDR-KO group. Taken together, this clearly indicates that the behavioural differences reported here for VDR-KO mice are not due to a possible difference in sensory abilities or emotionality/activity levels. The apparent lack of any motor deficit in the VDR-KO mice tested in the actometer and novel object-finding test suggests that their motor impairments do not appear in normal spontaneous behaviour, and can only be observed in more stressful situations requiring intense physical efforts (such as swimming or vertical screen retention). Together, our findings demonstrate that genetic ablation of VDR in mice is associated with specific impairments in motor performance in the vertical screen and swim tests, which are: (i) not accompanied by altered emotionality/activity or sensory/vestibular characteristics; (ii) can only be seen if strong physical stressors are applied; and (iii) occur only in VDR-KO animals.

How can loss of VDR lead to the impaired motor performance observed in VDR-KO in the present study? The widespread distribution of VDR in the brain and spinal cord suggests its functional properties in the nervous system including motor function [11,17,23]. Vitamin D has been implicated in a number of physiological processes in the brain, including the modulation of brain neurotransmitters such as acetylcholine and catheholamines [2,7], mediators that have long been known to be involved in the control of motor behaviour [18,25]. Given our findings in mutant mice, genetic ablation of VDR in the brain, especially in the structures involved in motor control, may disrupt brain vitamin D-VDR signalling pathways, which, associated with disturbed modulation of neurotransmitters in these regions, may cause the impaired motor performance reported in our experiments. Importantly, our data are consistent with earlier findings [1] linking vitamin D deficiency to inhibition of motor activity in animals (see also similar human data in [2,16,21]). As such, impairment of these functions in VDR-KO mice may be one of the reasons for the specific motor abnormalities, which we saw in our studies.

Moreover, since vitamin D plays an important role in the regulation of Ca^{2+} homeostasis, another possibility for poor motor performance in VDR-KO can be dysregulation of Ca^{2+} homeostasis, which is usually associated with vitamin D-related disorders [2,7]. To test this hypothesis, in a separate experiment we measured plasma Ca^{2+} level in VDR-KO mice. Indeed, despite a special rich Ca^{2+} diet, VDR-KO mice had generally lower plasma Ca^{2+} (2.04 ± 0.08 mmol/l, $P < 0.05$; $n = 6$), compared to 2.28 ± 0.10 mmol/l in the wild-type ($n = 3$) and 2.27 ± 0.48 mmol/l in heterozygous ($n = 7$) mice. Since Ca^{2+} is crucial for normal neuromuscular functions, its imbalance in the VDR-KO mice may affect their motor performance – the phenomenon, which we saw in our experiments. Furthermore, the motor abnormalities reported here for VDR-KO mice resemble several key symptoms of vitamin D-associated hypocalcemia including dyskinesia and neuromuscular weakness. This is also consistent with earlier observations [14,26] noting a general resemblance between VDR-KO mice and patients with vitamin D-linked rickets. Taken together, these data suggest that low Ca^{2+} level associated with ablated VDR may be a key pathological factor in developing the severe motor impairments found in our studies.

Finally, we note that the mice tested in the present study were 24–30 weeks old – the age, which can be classified as "adult-to-aged", according to the standard behavioural phenotyping protocols [4]. Although not directly tested in this study, this age-related aspect may be especially important to consider since VDR-KO mouse behaviour has not yet been studied in detail. For example, based on our general knowledge of age-related progression of motor disorders, it is possible to assume that motor deficits in VDR-KO mice are age-dependent, and that the age of the mice used here did affect the finding accordingly. As such, VDR-KO mice, especially when tested at different ages, may provide a very interesting model to study age-related motor deficits associated with vitamin D/VDR-related disorders. Since vitamin D-related disorders affect mostly elderly people [16,21], this important aspect of VDR-KO motor behaviour needs further experimental investigation, especially considering its potential clinical implications.

In conclusion, the results of the present study show motor abnormalities in mice associated with VDR genetic ablation and disturbed Ca^{2+} homeostasis. Our data confirm that vitamin D and VDR are crucial for motor activity, and disorders related to them significantly impair animal motor functions. These findings may be important given the emerging problem of vitamin D-related neural disorders, especially since large percentages of vitamin D-deficient population has been reported in many countries [8,20], and the growing number of motor disorders is being linked to deficit in vitamin D [2,7,16,21]. We suggest that the use of genetic animal models of vitamin D/VDR-related motor behavioural disorders, such as those reported here, may be a useful tool for further studies in this field.

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