Increased anxiety in mice lacking vitamin D receptor gene

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Vitamin D is a steroid hormone with many important functions in the brain, mediated through the vitamin D nuclear receptor. Numerous human and animal data link vitamin D dysfunctions to various behavioural disorders. To examine this problem, we studied whether genetic ablation of vitamin D receptors in mice may be associated with altered emotional behaviours. Here we show that the receptor-deficient mice demonstrate increased anxiety-like behaviours when subjected to a battery of behavioural tests. These studies suggest that vitamin D and its receptors are an important factor in the brain, whose imbalance may significantly affect emotional behaviour. *NeuroReport* 15:1271–1274 © 2004 Lippincott Williams & Wilkins.

Key words: Anxiety behaviours; Knock-out mice; Vitamin D receptors

INTRODUCTION

Vitamin D hormone is essential for growth and differentiation in a variety of organs, including the brain [1–3]. Numerous data suggest that vitamin D plays an important role in the brain through induction of many CNS genes, modulation of neuroprotection, neurotrophins release and activity of key neurotransmitter metabolism enzymes [1,2,4]. The functions of vitamin D are mediated through nuclear vitamin D receptors (VDR), which are widely distributed through the nervous system [1,3].

Evidence for the presence of vitamin D, the enzymes of its bioactivation and metabolism and functional VDR in the brain implies that this vitamin may have some function in the CNS as an autocrine or paracrine neurosteroid hormone [1]. VDR are found in key brain areas including the cortex, cerebellum and limbic system, all known to regulate behaviour [5–7]. In humans, vitamin D deficiency has long been known to be accompanied by irritability, depression, psychoses and defects in mental development [2]. The psychotropic mood-elevating effects of vitamin D have also been well documented in the literature [8]. In animals, vitamin D deficiency produces behavioural alterations including decreased exploration and maze performance [4]. Collectively, this suggests that vitamin D could be an important factor controlling behaviour in animals and humans. As such, various vitamin D and VDR-related disorders may therefore be a risk factor for abnormal emotional behaviour in both animals and humans.

We investigated this possibility in mice by examining whether genetic ablation of VDR may affect their emotional behaviours. In the present study we assessed the behavioural performance of VDR null mutant mice tested in several behavioural tests of anxiety.

MATERIALS AND METHODS

Subjects were 30 adult male mice (age 24–30 weeks) bred in the University of Tampere (Finland). VDR gene mutant mice were bred in the University of Tampere from the line initially generated in the University of Tokyo (Japan) [3,9]. 129 mouse substrain was used as a genetic background for these mutant mice. Ten null mutants were compared with ten control wild type (+/+) 129 mice and 10 heterozygous (+/−) mice. Mice of all three genotypes were littermates produced by heterozygous crosses. The mice were maintained in groups of two to three animals per cage in a virus/parasite-free facility (temperature $24 \pm 1^{\circ}C$, humidity $50 \pm 5\%$) and exposed to a 12:12 h light:dark cycle (lights on 07.00 h) with food and water *ad lib* (except food finding experiments, when in order to increase hunger, animals were food deprived for 10 h prior to testing). All testing was conducted between 17.00 and 19.00 h. On test days, animals were transported to the dimly lit laboratory and left undisturbed for 2 h prior to testing. General locomotor activity was measured for 5 min in a plastic actometer box ($30 \times 30 \times 30$ cm) with a floor divided into four squares (15×15 cm). Conventional measures were horizontal activity (the number of squares visited) 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 behavioural testing. 10 days later, the olfactory and visual abilities of mice were tested in an actometer. The latency (s) of finding food (cheese) and a novel object (5 cm metal sphere) were used as measures of olfactory and visual abilities. 10 days later, the animals' ability to keep their balance was assessed in the horizontal bar test. The horizontal bar (wooden bar 1 cm thick) was fixed to a platform that was elevated 30 cm from the floor.

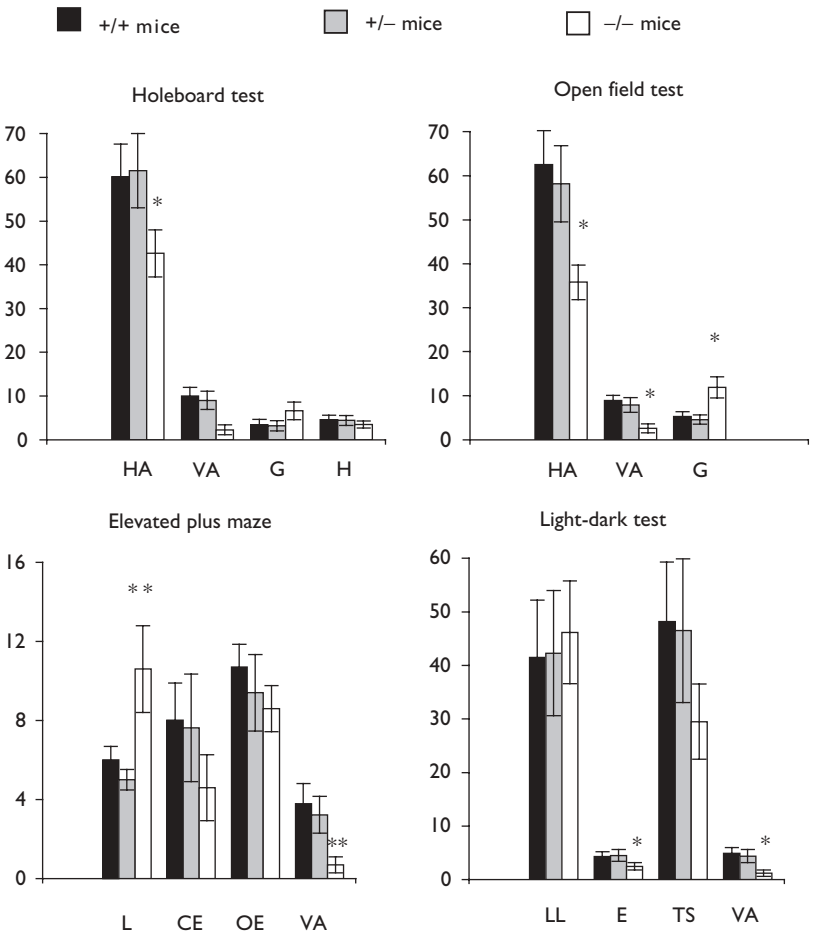

Fig. 1. Behaviours of VDR null mutant mice compared with wild type (+ +) and heterozygous (+/−) mice subjected to a battery of anxiety tests. HA, horizontal activity; VA, vertical activity; G, grooming duration (s); H, holes inspected; L, latency to leave the centre (s); CE, closed entries; OE, open entries; LL, latency to enter the light area; E, entries to the light area; TS, time spent (s) in the light area. *$p < 0.05$, **$p < 0.01$ significant difference between genotypes.

The mice were tested for 3 min and the time (s) the animals could keep their balance was measured. 10 days later, animal behaviour was assessed in a battery of anxiety tests including the open field, the holeboard, the elevated plus maze and the light-dark paradigms. Each test was performed following a 10 day recovery period. Behaviours were recorded for 5 min in each test by an experienced observer. The open field and holeboard tests were a plastic box ($45 \times 45 \times 45$ cm) with a floor divided into nine squares (15×15 cm). The holeboard removable floor had 64 holes (1 cm in diameter). Conventional measures in these tests were horizontal activity, vertical activity, total time spent grooming (s), and the number of holes inspected. The elevated plus maze was made from Plexiglas and consisted of two open arms (30×10 cm) and two enclosed arms ($30 \times 10 \times 10$ cm) extending from a common central region (10×10 cm) elevated to a height of 60 cm. Conventional measures were the initial latency (s) to leave the centre, the frequencies of open- and closed-arm entries (4-paw criterion), as well as vertical activity. The final test was the light-dark transition paradigm. The test was a Plexiglas box consisting of two exploratory units (transparent and black, $30 \times 30 \times 30$ cm each), interconnected by a sliding door. Conventional measures were the initial latency (s) to move from the dark to the light compartment, the number of the light entries (4-paw criterion), total time spent in the light compartment (s), and vertical activity. Between subjects, each apparatus was thoroughly cleaned (wet and dry cloths). Animal care and experimental procedures were conducted in accordance with the European legislation and the guidelines of the National Institute 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 U-test. In all tests, $p < 0.05$ was considered statistically significant.

RESULTS

Since genetically modified animals often demonstrate motor and sensory abnormalities which may non-specifically affect behaviour [10], we first tested the general locomotion and sensory abilities of VDR null mutant mice. Assessment of locomotor activity in the actometer test revealed that VDR null mutant, heterozygous and the wild type mice had

similar baseline levels of horizontal and vertical activity (horizontal activity: 37 ± 4, 42 ± 4 and 40 ± 5, respectively; vertical activity: 30 ± 3, 32 ± 3 and 35 ± 5, respectively). All three groups had unaltered major sensory (visual/olfactory) abilities as assessed on the food- and novel object-finding tests (latency to find food: 14 ± 3, 16 ± 3 and 17 ± 3 s, respectively; the latency to find novel object: 20 ± 4, 16 ± 3 and 17 ± 3 s, respectively). In addition, all three groups had unaltered vestibular system as assessed on the horizontal bar test (bar retention time: 125 ± 17, 104 ± 15 and 120 ± 21 s, respectively).

However, as can be seen in Fig. 1, marked behavioural alterations in VDR null mutants were found when the mice were subjected to a battery of anxiety tests. In the holeboard test, VDR null mutants demonstrated decreased horizontal and vertical activity (43 ± 5; $p < 0.05$, U-test and 3 ± 2; $p < 0.05$, U-test), compared with the wild type (60 ± 7 and 10 ± 2, respectively) and heterozygous mice (62 ± 8 and 9 ± 2, respectively).

In the open field, compared with the wild type and heterozygous control groups, VDR null mutants demonstrated decreased horizontal (37 ± 4 *vs* 63 ± 8 and 58 ± 9, respectively; $p < 0.05$, U-test) and vertical exploration (3 ± 1 *vs* 9 ± 1 and 8 ± 2, respectively; $p < 0.05$, U-test) as well as increased duration of grooming (12 ± 2 *vs* 5 ± 1 and 5 ± 1 s, respectively; $p < 0.05$, U-test). In the elevated plus maze, compared with the wild type and heterozygous groups, VDR null mutant mice demonstrated a dramatic decrease in vertical activity (0.7 ± 0.4 *vs* 3 ± 1 and 4 ± 1, respectively; $p < 0.01$, U-test) as well as longer latency to leave the central area (11 ± 1 *vs* 6 ± 1 and 5 ± 1 s, respectively; $p < 0.01$, U-test). As can be seen in Fig. 1, in the light–dark transition test, VDR null mutant mice showed significantly less exploration of the light compartment than did their littermates from both control groups (the time spent in the light compartment: 30 ± 7 s *vs* 48 ± 11 and 46 ± 13 s, respectively; $p < 0.05$, U-test; vertical activity in the light compartment: 1 ± 0.6 s *vs* 5 ± 1 and 4 ± 1 s, respectively; $p < 0.05$, U-test).

DISCUSSION

To the best of our knowledge, this paper is the first study characterizing altered behaviour of VDR null mutant mice. The importance of assessing the status of locomotor, vestibular and sensory systems is widely recognized as a key part of behavioural analysis of mutant animals [10]. VDR deficiency in knock-out mice has recently been reported to yield aberrant musculoskeletal development as well as rickets-like bone malformations [3,11]. Using different behavioural tests we show that, despite these abnormalities, VDR null mutant mice exhibited unaltered baseline motor activity, movements coordination and sensory abilities. This observation is in line with previously published works showing no obvious motor and neurological abnormalities in VDR-deficient mice [1].

However, our study revealed that VDR mutant mice displayed anxiety-like decreased exploration when subjected to anxiety tests. Notably, VDR null mutants react to stress by a robust decrease of vertical activity. Another specific feature of these mice was increased stress-induced grooming activity, as measured in the open field test (Fig. 1). Overall, these results indicate that VDR null mutant mice display a higher anxiety level compared to the wild type and heterozygous groups. Importantly, these findings are consistent with data from the literature showing that in rodents stress and anxiety are associated with decreased exploratory behaviours [10,12]. Furthermore, since alterations in vertical activity reflect emotional components of rodent behaviour [12,13], our results are in line with earlier studies [4] linking vitamin D deficiency to inhibition of open field vertical activity in rats. In addition, stress is also known to stimulate grooming behaviour in rodents [14], an effect which we also observed in VDR null mutant mice. Interestingly, the behavioural responses of heterozygous (+/−) mice were very similar to the wild type (+/+) mice. These behavioural observations are consistent with earlier data showing that heterozygous mice, possessing 50% of mutated VDR genes, show similar VDR gene mRNA expression, have no overt abnormalities and phenotypically are indistinguishable from the wild type animals [3,15]. Together, our findings demonstrate that genetic ablation of VDR in mice is associated with increased anxiety-like behaviours, and that this effect can only be seen in VDR null mutant animals.

How can loss of VDR lead to increased anxiety? Vitamin D has been implicated in a number of physiological processes in the brain, including the modulation of brain neurotransmitters such as acetylcholine and catecholamines [1,2], mediators that have long been known to be involved in the regulation of emotional behaviours. The widespread VDR distribution in the brain suggests its functional properties in the CNS [5–7]. Importantly, the highest brain VDR concentration has been found in the limbic system, the key emotiogenic brain structure, and its extensions in the brain [6,7]. Given our findings in mutant mice, we can suggest that genetic ablation of VDR in the brain, especially in the emotiogenic limbic structures, may disrupt vitamin D-VDR signalling pathways which, associated with disturbed modulation of neurotransmitters in these regions, may cause the increased anxiety level seen in our experiments.

CONCLUSION

Our results directly link VDR deficiency to increased anxiety symptoms and indicate that the vitamin D-VDR system significantly affects emotional behaviour. Defects of this system may directly lead to emotional disorders, as has already been speculated [1,2,8]. For the development of effective treatments for such disorders it is therefore necessary to increase our knowledge on the central function of this system.

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