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Behavioural Abnormalities in Mice with Partially Deleted Vitamin D Receptor Gene

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ACADEMIC DISSERTATION

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To my family

1. CONTENTS

2. ABSTRACT

Vitamin D is a seco-steroid hormone with several important functions in the nervous system, mediated through the nuclear vitamin D receptors (VDR). Numerous human and animal data link alterations in the vitamin D-VDR system to various behavioural disorders. Here we studied whether partial deletion of the VDR gene in mice may be associated with altered behavioural phenotypes. Overall, subjected to a battery of tests, VDR mutant mice showed increased anxiety, increased grooming activity, abnormal grooming sequencing, impaired swimming, aberrant nest-building and maternal behaviours, compared to their control littermates. Behavioural phenotypes of heterozygous mice were similar to the wild type control group, indicating that behavioural abnormalities associated with the lack of VDR are inherited as recessive. In contrast, olfactory, visual and balancing abilities, as well as sexual behaviours were unaltered in all three genotypes, suggesting that these domains are unaffected by the mutation. Overall, our results suggest that neurosteroid hormone vitamin D and VDR may play an important role in controlling various behaviours, further confirming the important role of the vitamin D system and VDR in the brain.

3. ABBREVIATIONS

aa	amino acid
A	angstrom
Ca	Calcium
CNS	central nervous system
HZ	heterozygous (+/-) mice
IU	International Units
K_d	dissociation constant
kb	kilobase
kD	kilodalton
MS	Multiple sclerosis
P	Phosphorus
PCR	Polymerase chain reaction
RXR	retinoic acid X receptor
UV	ultra-violet irradiation
VDR	nuclear vitamin D receptors
VDR KO	VDR knockout (-/-) mice lacking functional VDR
VDRm	putative membrane vitamin D receptors
VDRE	vitamin D response elements
WT	wild type (+/+) mice
1,25-D	1,25-dixydroxyvitamin D (calcitriol)
25-D	25-hydroxyvitamin D (calcidiol)
129S1	129S1/SvImJ mouse inbred strain

4. LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals:

I. Kalueff AV, Lou YR, Laaksi I, and Tuohimaa P (2004): Increased anxiety in mice lacking vitamin D receptor gene. Neuroreport 15(8), 1271-1274.

II. Kalueff AV, Lou YR, Laaksi I, and Tuohimaa P (2004): Impaired motor performance in mice lacking neurosteroid vitamin D receptors. Brain Res Bull 64(1), 25-29.

III. Kalueff AV, Lou YR, Laaksi I, and Tuohimaa P (2004): Increased grooming behavior in mice lacking vitamin D receptors. Physiol Behav 82(2-3), 405-409.

IV. Kalueff AV, Lou YR, Laaksi I, and Tuohimaa P (2005): Abnormal behavioral organization of grooming in mice lacking the vitamin D receptor gene. J Neurogenet 19(1), 1-24.

5. INTRODUCTION

Vitamin D is a seco-steroid hormone with multiple functions in the organism, including the regulation of mineral metabolism, cell proliferation, differentiation and apoptosis. Over the last decades, the brain is now emerging as a target tissue for this hormone, allowing to consider vitamin D as a neuro-active or neurosteroid hormone.

Biological effects of vitamin D in the brain include the regulation of brain development, neuroprotection, induction of activity of many CNS genes, modulation of the neurotrophin and cytokine networks, the activity of key brain enzymes, as well as possible psychotropic (anticonvulsant, antidepressant-like) effects.

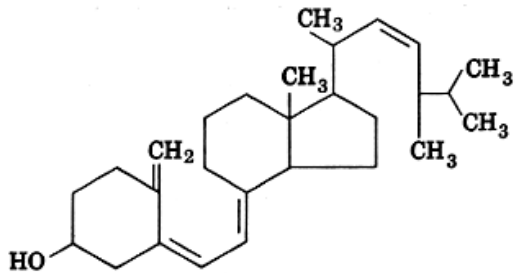
Many of biological effects of vitamin D are mediated through the nuclear vitamin D receptor (VDR), a member of a common family of steroid receptors. Since mutant mice lacking functional VDR are currently available for biomedical research focusing on the role of vitamin D in different organs, these animals represent a particularly useful tool to study the effects of vitamin D in the brain.

Various vitamin D/VDR-related dysfunctions have already been reported to lead to different behavioural disorders in both animals and humans. Therefore, behavioural analysis of the effects of the VDR gene partial deletion in a mouse model represents an important task, enabling our better understanding of the role of vitamin D in the brain. The present study was undertaken to characterize in detail the behavioural phenotypes of mutant mice lacking functional VDR.

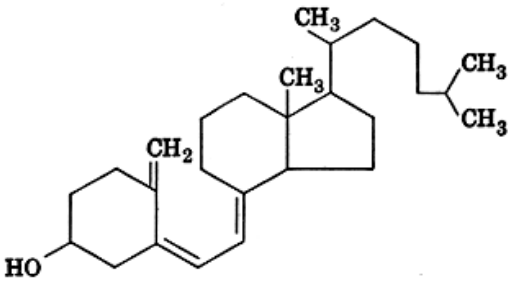
6. REVIEW OF THE LITERATURE

6. 1. THE VITAMIN D ENDOCRINE SYSTEM

The discovery of vitamin D (calciferol) and the elimination of rickets is one of the major medicine's achievements (DeLuca 2004). Vitamin D is a fat-soluble seco-steroid hormone, existing in two main (almost equally potent) forms - D_2 (ergocalciferol) and D_3 (cholecalciferol, Fig. 1), (Malloy et al. 1999; Dusso et al. 2005). The chemical structure of vitamin D was determined in the 1930s by Adolf Windaus. Vitamin D_2, which could be produced by UV irradiation of plant sterol ergosterol, was chemically characterized in 1932. Vitamin D_3, synthesized in skin during photolysis of 7-dehydrocholesterol, or ingested with food (Carswell 1997; Garcion et al. 2002), was first isolated in 1936 from fish oil by Hans Brockmann.



Vitamin D_2



Vitamin D_3

Figure 1. Chemical structure of vitamins D_2 and D_3

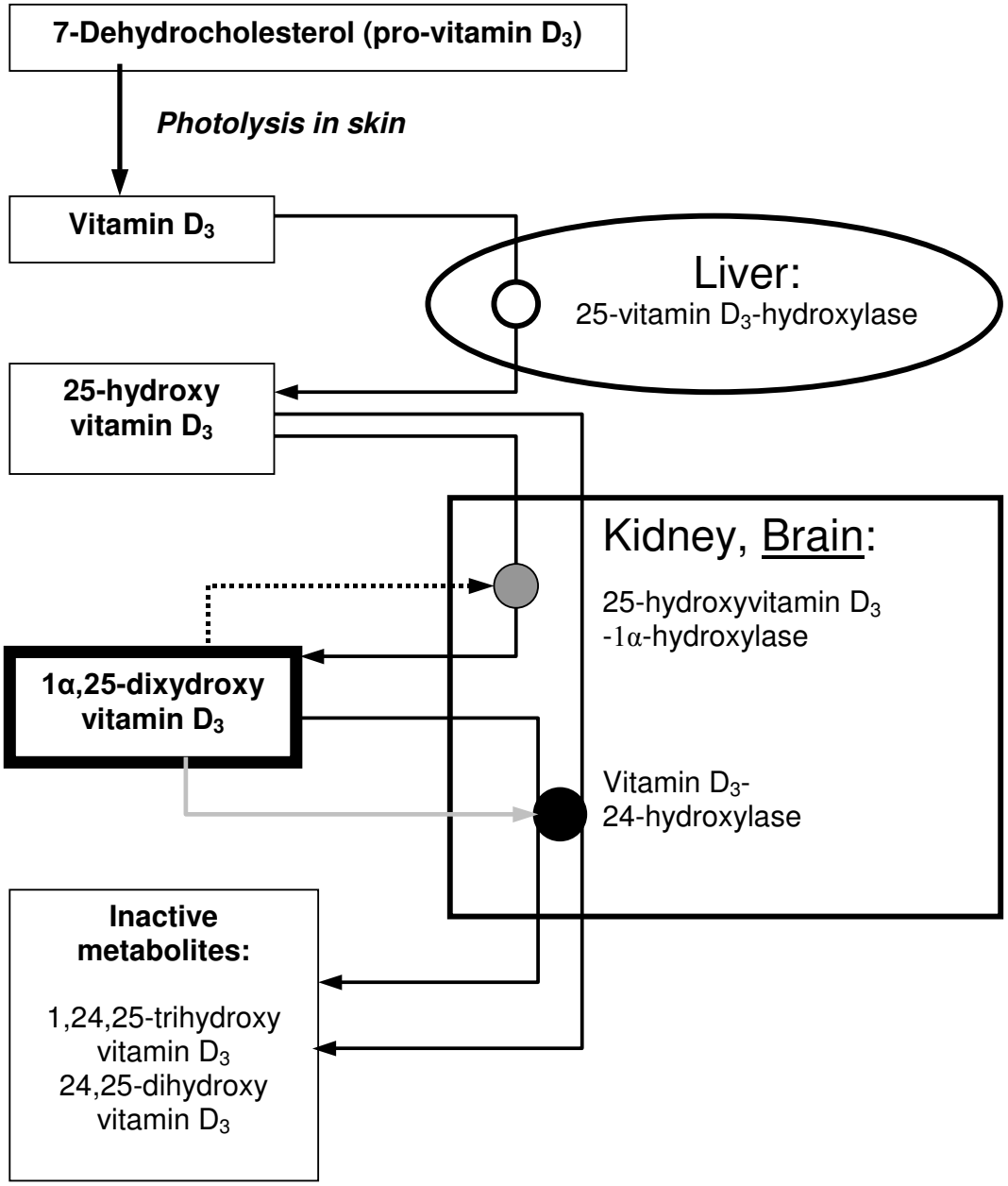


Figure 2. Synthesis and metabolism of vitamin D. Disrupted line: inhibition, grey line - activation of enzyme activity by vitamin D metabolites

25-D is the major circulating form of vitamin D, and has some activity in different tissues (St-Arnaud 1999; Lou et al. 2004ab; Tuohimaa et al. 2005). Production of 25-D is not significantly regulated, being primarily dependent on substrate concentration. 1,25-D is the

most active hormonal form of vitamin D (Kato et al. 1998, 1999). Together with 25-D, lipophilic 1,25-D circulates in blood as complexes with vitamin D-binding protein, albumin, α-fetoprotein, and lipoproteins (rev.: Chatterjee 2001; Kalueff et al. 2004a). Activity of kidney 25-hydroxyvitamin D-1α-hydroxylase is down-regulated by 1,25-D (Fig. 2), thus tightly controlling the concentrations of this biologically active substance in the blood. Low serum Ca and P, calcitonin, estrogen and insulin-like growth factor induce 25-hydroxyvitamin D-1α-hydroxylase, whereas high Ca and P suppress its enzymatic activity (rev. Ahonen 2002).

In kidneys, both 1,25-D and 25-D are catabolised by oxidation of the side chain by vitamin D-24-hydroxylase, leading to formation of inactive metabolites 24,25-dihydroxyvitamin D and 1,24,25-trihydroxyvitamin D (calcicetrol), Fig. 2 (Norman et al. 2001), although some activity has been recently suggested for 24,25-dihydroxyvitamin D (Nemere 1999; St-Arnaud 1999; Scott et al. 2005). This catabolic enzyme is down-regulated by high Ca and P, and up-regulated by 1,25-D, implying its important role in maintaining physiological concentrations of 1,25-D (Jones et al. 1998; Ahonen 2002; DeLuca 2004).

The most important biological function of vitamin D is mineral homeostasis, where together with other endocrine hormones it is involved in Ca metabolism by regulating renal and intestinal Ca transport and bone mineralization (Issa et al. 1998; Haussler et al. 1998; Li et al. 2002, 2004). Several additional functions of this hormone include the regulation of blood pressure (via negative regulation of renin-angiotensin system), the immune system, tissue proliferation, differentiation and apoptosis, as well as anti-cancer protection (Kinuta et al. 2000; Ylikomi et al. 2002; Li et al. 2004; Lou et al. 2004b; Uitterlinden et al. 2004ab; Tuohimaa et al. 2005).

6.2. VITAMIN D RECEPTORS

1,25-D regulates the expression of numerous target genes through the nuclear vitamin D receptor (VDR), serving as a ligand-inducible factor (Issa et al. 1998; Jones et al. 1998; Malloy et al. 1998; Collins et al. 2005). 1,25-D is the main ligand for VDR, easily penetrating the plasma membrane of its target cells (Juntunen 2002; Carlberg 2004). Upon

binding 1,25-D, the VDR undergoes a conformational change and forms a complex with a retinoid X receptor (RXR, Juntunen 2002), Fig. 3. Although vitamin D and retinoic acid are very different in their chemical structure, their nuclear receptors show a substantial homology in both DNA- (45-60%) and ligand- (15-30%) binding domains (Chatterjee 2001). VDR–RXR complex binds to DNA elements in the promoter regions of target genes described as vitamin D response elements (VDRE, usually found within 1 kb of the start site of the target gene), thus, controlling the rate of gene transcription (Juntunen 2002; DeLuca 2004; Hoenderop and Bindels 2005).

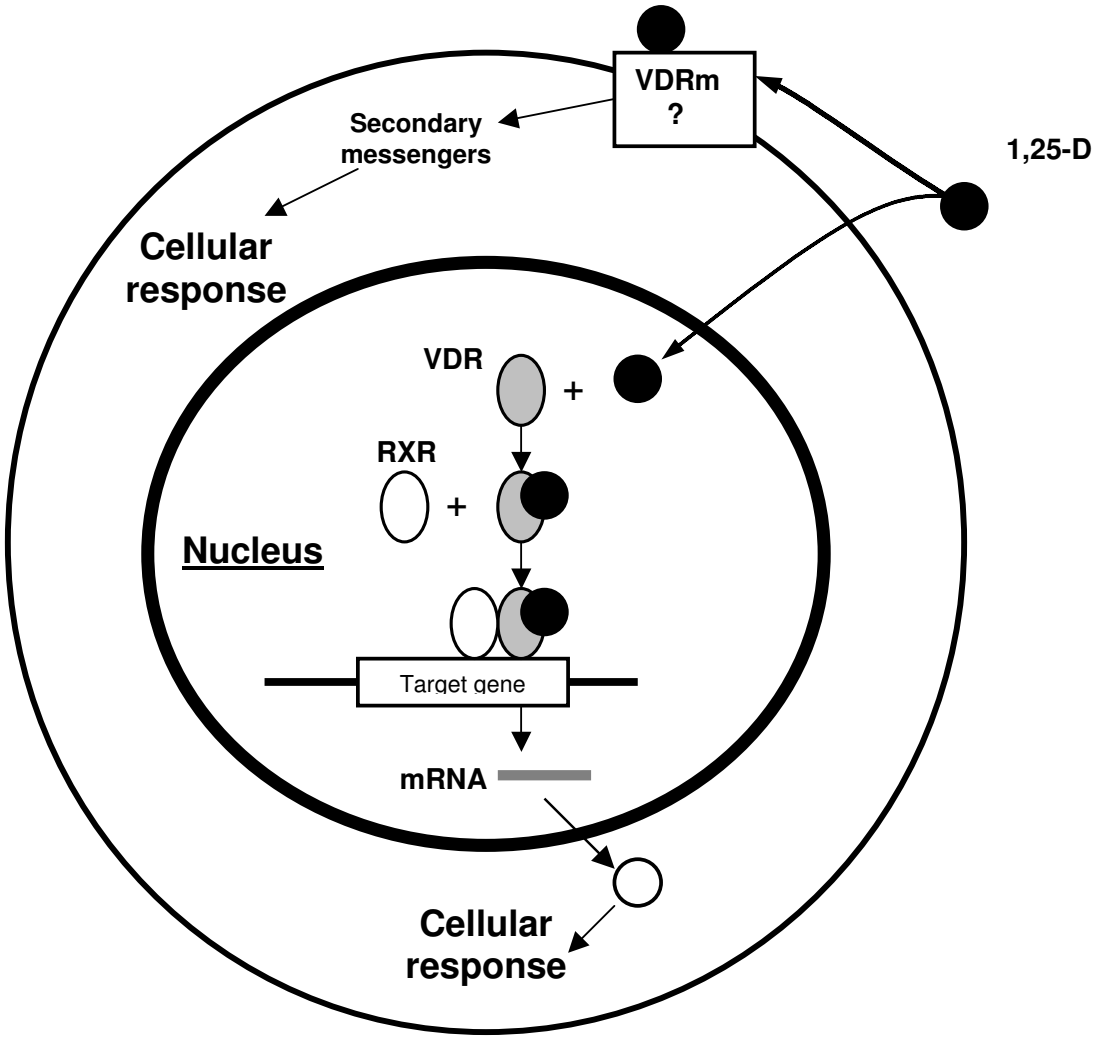


Figure 3. Mechanisms of molecular action of vitamin D

Table 1. Domain structure of the vitamin D nuclear receptor (VDR; Malloy et al. 1999; Ylikomi et al. 2002; Sulton and MacDonald 2003; Carlberg 2004)

Domain	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 (main role: the first zinc finger), also participates in nuclear localization and dimerization with the retinoic acid X receptor (RXR, main role: the second zinc finger)
D (≈30 aa)	Highly flexible "hinge" region	Confers rotation flexibility between C and E domains to allow simultaneous VDR dimerization and DNA-binding
E, or E/F (≈310 aa)	Ligand-binding domain (similar to other nuclear receptors, especially RXR); organized in 12/13 α-helices and 3 β-sheets forming a hydrophobic pocket (K_d for 1,25-D is about 0.5nM, with approximate volume of 620 A^3). Also includes the ligand-dependent activation function-2 (AF-2, ≈18 aa, a short 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 (SRC family, DRIP multiprotein complex, several other proteins interacting with and modulating the VDR transactivation/transrepression). AF-2 fragment of this domain is crucial for ligand-activated transcription, "locking up" ligands in the binding pocket

VDR belongs to a common family of nuclear receptors, which also includes steroid, retinoic acid and various orphan receptors (Cornet et al. 1998; Norman et al. 2001; Collins et al. 2005). It is a 50-60 kDa protein (depending on species), consisting of several functional domains, typical for all steroid receptors, and responsible for ligand and DNA binding, heterodimerization, nuclear localization and transcriptional activation (Table 1), also see (Norman et al. 2002). Cloning and sequencing of the VDR gene (≈65 kb) has been carried out in some species including chickens, humans, rats and mice. 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).

The E-domain of VDR is responsible for ligand binding, and is formed by 12 α-helical regions arranged into three β-layers, forming the ligand-binding pocket (Malloy et al. 1998). The mechanism of the genome effects of vitamin D is similar to that of all other steroid hormones (rev.: Losel and Wehling 2003), Fig. 3. DNA-complexed VDR acts as a molecular switch of nuclear 1,25-D signalling by transmitting its activation status to different chromatin loci containing its target genes (Fig. 3). The VDRE of these genes consist of two hexameric motifs in a directly repeated or inverted palindromic arrangement (Carlberg 2003). Approximately 0.5% of the human genome (about 200 genes) are estimated to be primary targets of 1,25-D, although via various mechanisms the VDR appears to interfere in the regulation of even more genes (Carlberg 2003). Recently, >900 vitamin D-regulated genes have been reported in the literature (Wang et al. 2005).

VDR genes are highly polymorphic, and their variations occur frequently in the population (including missence and nonsence mutations, splice site mutations, and partial deletion), affecting DNA-binding, nuclear localization, ligand-binding, heterodimerization and other functions of the VDR), underlying various vitamin D-related dysfunctions (leading to partial or total hormone resistance and rickets), and demonstrating the variability in the vitamin D/VDR endocrine system (Malloy et al. 1997, 2002; Uitterlinden et al. 2004ab).

The rapid response to vitamin D uses non-genomic signal transduction pathway, believed to occur via putative membrane receptors for 1,25-D (VDRm, Fig. 3) (Jia and Nemere 1999;

Norman et al. 1999, 2001, 2002; Boyan et al. 2002ab, 2003). Although VDRm-like proteins were found in several tissues, including brain (Jia and Nemere 1999), their functions and properties are not yet well understood (Hoenderop and Bindels 2005). It is suggested that VDRm is a 60 kD protein with a high affinity to 1,25-D (K_d = 0.5-0.7 nM, Brown et al. 1999; Norman et al. 2002). However, the VDRm has not yet been cloned, and its domain structure remains unknown (rev.: Kalueff et al. 2004a). It has been recently suggested that VDR and VDRm may represent the same receptor protein, responsible for both genomic and non-genomic effects of vitamin D (Huhtakangas et al. 2004); in line with this notion, several recent studies have confirmed the importance of classic VDR for rapid (non-genomic) responses (Nguyen et al. 2004; Zanello and Norman 2004). Fast non-genomic effects of vitamin D occur within seconds, and their signal transduction is thought to involve the formation of second messengers, such as cyclic nucleotides, diacylglycerol, inositol-trisphosphate, and arachidonic acid (Nemere and Campbell 2000; Boyan et al. 2002b, 2003; Schwartz et al. 2003).

6.3. THE VITAMIN D SYSTEM AND THE NERVOUS SYSTEM

Although brain has long been considered as a target tissue for vitamin D (Stumpf et al. 1982, 1988; Stumpf and O'Brien 1987), the neurobiological role of this hormone was confirmed when the regulatory effects of 1,25-D on neuronal choline acetyltransferase (Sonnenberg et al. 1986) and nerve growth factor (Jehan et al. 1991; Neveu et al. 1991; Wion et al. 1991) were reported. Evidence for the presence of vitamin D, its binding sites, the enzymes of its bioactivation and metabolism (Fig. 2) and functional VDR in the brain of animals and humans implies that this vitamin may serve in the CNS as an autocrine or paracrine neuroactive hormone (Musiol et al. 1992; Magrassi et al. 1992; Stumpf et al. 1992; Sutherland et al. 1992; Carswell 1997; Perez-Fernandez et al. 1997; Garcion et al. 2002; Eyles et al. 2005). Physiological concentrations of 1,25-D in the brain are around 10 pM; this hormone can cross the blood-brain barrier and binds to its receptors in the brain (rev.: Kalueff et al. 2004a). VDR, binding 1,25-D, have been found in brain neurons, glial cells, brain macrophages, spinal cord, and the peripheral nervous system (Neveu et al. 1994; Stumpf et al. 1988; Cornet et al. 1998; Veenstra et al. 1998; Glaser et al. 1999; Prifer et al. 1999; Baas et al. 2000). In addition, 1,25-D has been reported to activate the expression of

VDR in Schwann cells (Cornet et al. 1998). Likewise, VDRm have also been identified in the brain, where these receptors have been reported to bind 1,25-D (Nemere and Campbell 2000), suggesting that acting via VDR and VDRm, vitamin D may modulate neurotransmitter release and neuronal activity in analogy to other steroid hormones (Cornet et al. 1998; Jia and Nemere 1999). Taken together, these findings imply the important role of this hormone and its receptors in both the central and the peripheral nervous systems. Based on our present knowledge of its functioning and synthesis in the nervous system, neuroactive steroid vitamin D can also be called neurosteroid hormone (McGrath et al. 2001, 2004; Rupprecht and Holsboer 2001).

Due to its well-known antiproliferative activity, vitamin D is an important regulator of brain development and differentiation, and its deficiency leads to different brain anomalies, such as longer cortex and lateral ventricles but thinner neocortex and narrower anterior comissure (Eyles et al. 2003; Ko et al. 2004; Mackay-Sim et al. 2004; Feron et al. 2005). Interestingly, vitamin D depletion led to more proliferating and fewer apoptotic neurons, but did not affect brain VDR levels, suggesting that the regulation of VDR in the CNS may be more complicated than it was previously recognized (Eyles et al. 2003; Ko et al. 2004; McGrath et al. 2004).

Vitamin D and VDR are responsible for several important brain functions, including neuroprotection, occurring via multiple mechanisms, such as: 1) reduction of Ca toxicity by stimulation of expression of Ca-binding proteins (calbindins, parvalbumin) or inhibition of expression of L-type Ca channels, 2) modulation of glutathione metabolism, 3) possible direct antioxidant-like effects, 4) reduction of nitric oxide synthesis, 5) induction of neurotrophins and neurogenesis, 6) modulation of cytokine release as an endogenous neuroimmuno-supressing agent (Cantorna et al. 1998; Bemiss et al. 2002; Garcion et al. 2002; Brown et al. 2003; Kalueff et al. 2004a). In addition, 1,25-D has been shown to exert robust anti-ischemic effects in the brain cortex, accompanied by significant reduction in heat shock proteins 27 and 32 (Losem-Heinrichs et al. 2004, 2005), up-regulation of glial heme oxygenase-1 (metabolizing and detoxifying free heme to endogenous antioxidants biliverdin and bilirubin) and down-regulation of glial fibrillary acidic protein (a sensitive marker for reactive gliosis) (Oermann et al., 2004).

Well-known immuno-modulating properties of vitamin D (Bemiss et al. 2002) imply its potential role in the neuro-immune interactions. Low vitamin D status has been implicated in the etiology of autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis, insulin-dependent diabetes mellitus, and inflammatory bowel disease (Meehan and DeLuca 2002; Cantorna and Mahon 2004; DeLuca 2004; Chaudhuri 2005). The prevalence of MS is highest where environmental supplies of vitamin D are lowest, strengthening the potential role of this hormone as a natural inhibitor of MS (Hayes 2000; Munger et al. 2004; Van Amerongen et al. 2004). The systemic and local increase in the expression of several anti-inflammatory cytokines by 1,25-D are responsible for the ability of vitamin D to block, in a Ca-dependent manner, experimental encephalomyelitis, an animal model of MS (Cantorna et al. 1998, 1999). Anti-MS effects of vitamin D may involve paracrine or autocrine metabolism of 25-D by target cells, altered macrophage, dendritic and T-cells functions, their sensitization to apoptotic signals, as well as some action on the CNS component of MS pathogenesis (Spach et al. 2004; Van Amerongen et al. 2004), and generally seem to be associated with genomic VDR-mediated pathways (Cantorna 2000; Meehan and DeLuca 2002).

A growing body of literature suggests the link between vitamin D-related disorders and epilepsy (Maeda and Ikeda 1986; Riancho et al. 1989; Carswell 1997; Ali et al. 2004; Holick 2005). Seizures due to low vitamin D, common in patients with hereditary or nutritional rickets, are reduced by vitamin D, underlying the possibility of anticonvulsant properties of this hormone (Hess 1929; Christiansen et al. 1974; Gupta and Grovel 1977; Offermann et al. 1979; Oki et al. 1991; Hoecker and Kanegaye 2002; Johnson and Willis 2003; Armelisasso et al. 2004). Moreover, anticonvulsant effects of 1,25-D, injected systemically or directly into the brain, have been reported in rats (Siegel et al. 1984; following the electrical stimulation of the dorsal hippocampus), and in mice (Kalueff et al. 2005b; during convulsant (pentylenetrazole)-induced seizures), thus further confirming the role of the vitamin D/VDR system in epilepsy.

In 1997, Prufer and Jirikowski reported neuronal co-localization of VDR with oxytocin, suggesting a direct genomic action of this steroid on oxytocin expression, similar to other

effects of steroid hormones on peptidergetic systems. In line with this, numerous data link the vitamin D system to the regulation of behaviour. VDR are found in key brain areas including the cortex, cerebellum and limbic system, all known to regulate behaviour (Prufer et al. 1999; Walbert et al. 2001; Langub et al. 2001). In humans, vitamin D deficiency has long been known to be accompanied by irritability, depression, psychoses and defects in mental development (Carswell 1997). The psychotropic mood-elevating effects of vitamin D have also been well-documented in the literature (Stumpf and Privette 1989; Lansdowne and Provost 1998; Gloth et al. 2001). In animals, vitamin D deficiency produces behavioural alterations including decreased exploration and maze performance, hyperlocomotion and impaired pre-pulse inhibition and memory (Altemus et al. 1987; Burne et al. 2004ab; Becker et al. 2005), while neonatal treatment with vitamin D has been shown to affect sexual behaviours in rats (Mirzahosseini et al. 1996). Collectively, this suggests that vitamin D could be an important factor controlling brain functions in animals and humans.

6.4. MICE LACKING FUNCTIONAL VDR

Mice with genetically impaired VDR (knockout mice, KO) are currently available for biomedical research focusing on the biological functions of vitamin D and VDR (Kallay et al. 2001; Song et al. 2003; Bula et al. 2005ab). Several groups have independently generated VDR KO mice by targeted disruption of this gene. Yoshizawa et al (1997) ablated the 1.1-kb fragment containing exon 2, which encodes the first zinc finger of the DNA-binding domain of the VRD gene (Tokyo mice). Li et al. (1997) ablated a fragment spanning exons 3-5 and encoding the second zinc finger (Boston mice). In addition, Erben et al (2002) have recently generated mice expressing non-functional VDR without the first zinc finger (Munich mice), while Van Cromphaut et al (2001) deleted a fragment encompassing exons 1 and 2 (Leuven mice). All these mice display similar physiological phenotypes, with pronounced rickets, resembling human hereditary vitamin D-resistant rickets type II (Li et al. 1997). VDR KO mice demonstrate alopecia, hypocalcemia, hypophosphatemia, elevated plasma vitamin D (due to the lack of VDR-mediated negative feed-back), impaired reproductive system, muscular and skeletal abnormalities, and normally die by 15 weeks (Yoshizawa et al. 1997; Li et al. 1997), Fig. 4.

Although rescue by high Ca/P diet has been shown to completely prevent most of these physiological anomalies in VDR KO mice, several aberrant features were not corrected in these mice, including alopecia and reduced brain levels of calbindin D9k, implying direct VDR-dependent Ca/P-independent mechanisms (Li et al. 1998ab; Johnson and DeLuca 2001). Since the absence of functional VDR results in the target tissue insensitivity to genomic effects of vitamin D, the analysis of the behaviour of these mutant mice seems to be an important tool to assess the role of the vitamin D/VDR system in the brain. Ca/P-rich diet has been shown to dramatically extend the life-span of VDR KO, thus allowing us to study adult VDR KO mice, focusing on their behavioural phenotypes.

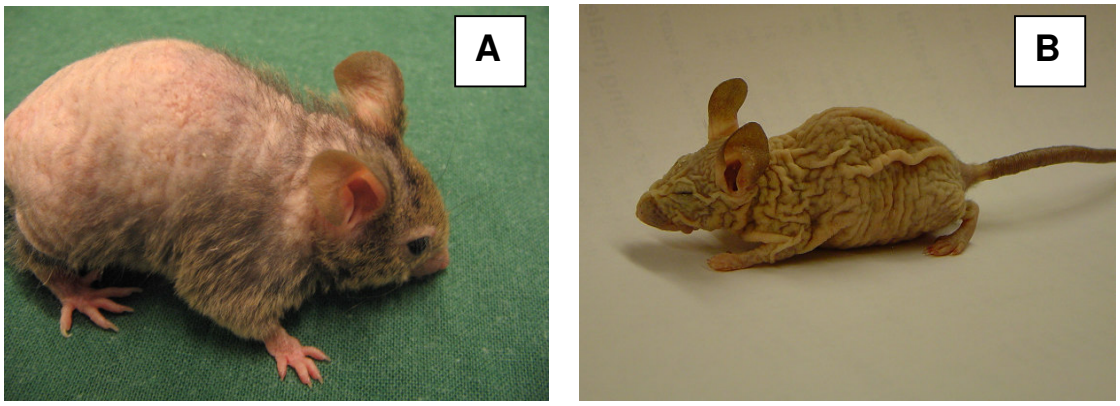


Figure 4. Phenotype of VDR knockout mice: A – adult (4 month old), B – ageing (12 months old) mice. Note progressing alopecia and lacking whiskers

Although it was generally accepted that Tokyo VDR KO mice are "lacking VDR" (Yoshizawa et al. 1997), recent studies (Bula et al. 2005ab) have shown that these mice, in fact, express truncated VDR, retaining full ligand-binding ability, but not DNA-binding activity (a situation equivalent to Boston mouse strain, displaying similar phenotypes resulting from the inactive VDR). We shall note that DNA binding in VDR is crucial for the regulation of a network of target genes (Carlberg 2003, 2004), and therefore partial deletion of VDR D-domain in Tokyo VDR KO may be responsible for their numerous physiological abnormalities reported in the literature. However, ligand-binding domain *per se* may be

responsible for several functions of VDR, not requiring the presence of DNA-binding domain (e.g., Scott et al. 2003; Vertino et al. 2005). It is therefore possible that some VDR-mediated functions may be retained in our mice, as well as in other VDR KO strains with partially deleted (but not totally ablated) VDR. Clearly, this notion requires further experimental investigation in detail (Bula et al. 2005ab). Nevertheless, the extent of physiological anomalies in all these KO mice allows to conclude, in line with Yoshizawa et al. (1997) and our general knowledge of nuclear receptors functioning, that disrupted DNA-binding ability leads to major disturbance in the vitamin D-VDR signaling pathways in these mutant mice. Interestingly, since recent data suggest that VDR and VDRm may represent the same protein (Huhtakangas et al. 2004), and that functional VDR are required for both genomic and non-genomic responses (Zanello and Norman 2004), it is possible to assume that both signaling mechanisms may be affected in VDR KO mice, jointly contributing to aberrant physiology of these mutants.

In any case, the unique physiology of these mice, as mentioned earlier (the lack of VDR in combination with elevated plasma 1,25-D), makes them particularly suitable to dissect different mechanisms of vitamin D action in the regulation of behaviour. For example, unimpaired behavioural phenotypes of these mutants would indicate VDR-independent mechanisms of action of this hormone. In contrast, altered behaviours in these mice would support the important role of VDR and VDR-mediated mechanisms in the regulation of behaviour.

6.5. BEHAVIOURAL PHENOTYPING OF MUTANT MICE

During the past decades, a remarkable link has been formed among physiology, molecular biology, and behavioral biology (Gerlai 1996; Zeiss 2004). The species-typical behaviors have been extensively studied in mice, and consist of eating-related movements, aggressive behaviors, sexual, parental and maternal behaviors, self- and heterogrooming, food hoarding, burrowing, nesting and exploration (Chen et al. 2005). In-depth analyses of these behaviours (or behavioural domains) in various mutant or transgenic mice represent an essential part of their behavioural phenotyping (Crawley and Paylor 1997; Crawley 1999, 2000).

Traditional and well-validated exploratory-based behavioural tests (Table 2), widely used in behavioural neurogenetics in rodents, include the open field, holeboard, elevated plus maze and the light-dark box, based on the balance between the motivation to explore and the fear of novelty (Golani et al. 1993; Rodgers and Cole 1994; Rodgers and Johnson 1996; Espejo 1997; Lapin 2000; Belzung 2001; Belzung and Griebel 2001; Choleris et al. 2001; Cruzio 2001; Drai et al. 2001; File 2001; Wall and Messier 2001; MPD 2001; Clement et al. 2002; Crabbe et al. 2002; Rodgers et al. 2002). Novel odor and novel object exposure tests represent another group of useful tests, measuring both the sensory functions and anxiety (neophobia) in animals (Kemble and Bolwahnn 1997; Kalueff and Tuohimaa 2004b).

Table 2. Summary of several behavioural models and indices commonly used in behavioural phenotyping of mutant or transgenic mice (Belzung 1999; Crawley 1999, 2000; Choleris et al. 2001; Kalueff and Tuohimaa 2004b)

Open field test (anxiety and activity test) • Defecations, urinations • Horizontal activity (total distance traveled or squares crossed) • Speed of movements • Distance in the inner/outer area • Number of squares crossed in the inner area • Self-grooming latency, duration, frequency • % of interrupted (aborted) or incorrect grooming • Average duration (s) of a single grooming bout • Vertical activity (wall leaning, unsupported vertical rears)
Actimeter (activity test) • Defecations, urinations • Horizontal activity (squares crossed) • Self-grooming latency, duration (s), frequency • Vertical activity (rears; as in the open field)

Novel object test (olfactory functions and anxiety test)

- Approach latency (s)
- Number of contacts
- Duration (s) of exploration of the novel object

Elevated plus maze (anxiety test)

- Latency to leave the center (s)
- Total arms entries (4 paws), enclosed arms entries
- Open arms entries (4 paws) and rears (2 paws)
- Time spent in the open arms, in the enclosed arms
- % Open arms entries, % Time spent in the open arms
- Head dips (looks down)
- Defecations, urinations
- Self-grooming latency, duration (s), frequency
- Central platform crossings and time (s) spent

Holeboard test (anxiety and activity test)

- Head dipping (hole poking) latency, number, duration
- Defecations, urinations
- total distance traveled/squares crossed
- distance/squares crossed in the inner area, distance in the outer area
- self-grooming, vertical rears – as in the open field test

Light/dark box (anxiety test)

- Light box entries number (4 paws)
- Light box time spent (s)
- Light box looks out (rears) number (2 paws)
- Duration of light box rears (s)
- Latency of the first rear and entry (s)
- Vertical activity in the light box (c)
- Urinations, defecations, grooming – as in the open field

Assessment of grooming behaviour is an essential part of behavioural phenotyping of various inbred and mutant mouse strains (Bolivar et al. 1996; Cromwell et al. 1998; Kalueff and Tuohimaa 2004ac). Examination of neurological phenotypes in various mice also represents a standard practice in their behavioural phenotyping (Thifault et al. 1996; Crawley and Paylor 1997; Hunter et al. 2000; McFadyen et al. 2003). Moreover, since mice of both sexes show nest-building activity, its assessment may be a useful tool in behavioural phenotyping of mutant mice (Sieber et al. 1980; Bulloch et al. 1982; Crawley 2000; MGI 2001; Moretti et al. 2005). Likewise, phenotyping of maternal behaviour, categorized into direct (direct contact with the offspring) and indirect (i.e., maternal nest-building), also represents an important area in behavioural neurogenetics (MGI 2001; Libhaber and Eilam 2002; Caldji et al. 2004; Jin et al. 2005).

In general, the use of these extensive methods not only allows the researcher to assess in detail the role(s) of different genes in the regulation of mouse behaviours (Cruzio 2001; Rodgers 2001; Lipp and Wolfer 2003), but also serves the main goal of animal behavioural modeling – creation of the valid animal models of human behavioural disorders (McKiney 1984, 2001; Overall 2000; Sarter and Bruno 2002).

7. AIMS OF THE STUDY

Brain is a target tissue for vitamin D, where nuclear VDR mediate many biological effects of this hormone. This study was aimed to characterize behavioural phenotypes of "Tokyo" mice lacking functional VDR (due to deletion of DNA-binding domain of the receptor).

The specific aims of the present study were:

1. to assess motor and activity behavioural profiles of these mice,
2. to examine their anxiety/emotionality in a battery of tests,
3. to study possible motor-sensory anomalies due to VDR partial deletion,
4. to assess grooming activity and its sequencing in VDR KO mice,
5. to assess several additional aberrant behaviours in these animals.

8. MATERIALS AND METHODS

8.1. ANIMALS

VDR KO mice were bred in the University of Tampere from the line initially generated in the University of Tokyo (Yoshizawa et al. 1997). Subjects were adult HZ, WT or KO mice, which were littermates produced by 4-5 heterozygous crosses and born in a ratio of approximately 1:2:1 (WT, HZ, KO). 129S1/SvImJ (129S1) mouse strain was used as genetic background for these KO mice. For genotyping, tail clips were taken for Polymerase chain reaction (PCR) on DNA prepared from tail tissue. Four primers were used to amplify a 130-bp VDR band and a 150 or 450-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. All mice used in the present study were maintained in a virus/parasite-free facility and exposed to a 12-h light, 12-h dark cycle. The animals were experimentally naïve and housed in groups of four in individual transparent cages (26 x 12 x 14 cm), with food and water freely available (except food-finding experiments). To normalize mineral homeostasis in these mice, a special high-Ca, high-P, high-lactose diet, containing 2% Ca, 1.25% P and 20% lactose, supplemented with 2.2 IU vitamin D/g (Lactamin AB, Sweden; Dardenne et al. 2003) was given to these mice from weaning.

8.2. PROCEDURES

All experiments were performed between 14.00 and 19.00 h. On test days, animals were transported to the dimly lit laboratory and left undisturbed for 2 h prior to testing (I). General locomotor activity was measured for 5 min in a plastic actimeter box (30x30x30 cm) with a floor divided into 4 squares (15x15 cm; I,II). 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 actimeter. 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. 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 time. Behaviours were recorded for 5 min in each test by an experienced observer. The open field and holeboard tests were a plastic box (45x45x45 cm) with a floor divided into 9 squares (15x15 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 (30x10 cm) and two enclosed arms (30x10x10 cm) extending from a common central region (10x10 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. 10 days later, the animals were exposed to the light-dark transition paradigm. The test was a Plexiglas box consisting of two exploratory units (transparent and black, 30x30x30 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.

The mouse motor performance (II) was assessed in the vertical screen test. Each 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.

Twenty days later, animal motor performance was assessed in the swim test (II) in 6 VDR KO and 5 WT and 5 HZ mice. 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 (II).

In our grooming experiments (III), we assessed the mouse grooming in a battery of behavioral tests including actimeter, open field, hole board, elevated plus maze tests (as described previously), and 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. 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.

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 (III).

In grooming analysis experiments (IV), we examined two types of grooming behaviour: (i) spontaneous (novelty-induced) and (ii) "artificial" (swim-induced). To evoke spontaneous grooming, the mice were placed individually in a clean plastic observation box (30 x 30 x 30 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 ± 1 ^{o}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.

In a separate experiment we analyzed behavioural patterning of a non-grooming complex behaviour (mating) in WT, HZ and KO mice (IV). For this, following a 10-day acclimation time, 6 mice of each genotype used in the previous experiments, were tested in the actimeter test. To induce sexual behaviour, we paired sexually naïve males with a WT female in estrus, assessing the following patterns for 10 min: 1) non-sexual behaviour (NSB, any behaviour, 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 analyzed for each pattern. In addition, we analyzed 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.

The following 6-point scaling system, based on our grooming analysis algorithm (Kalueff and Tuohimaa 2004a) was used to assess grooming microstructure (patterns): 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 categorized 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 between grooming patterns were assessed using the grooming analysis algorithm. “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 analyzed using the transition matrix, and the percentages of incorrect transitions were calculated for all three genotypes. 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. Complete/incomplete and interrupted/uninterrupted grooming bouts were analyzed in this study, and the percentages of incomplete bouts and interrupted bouts were calculated for all genotypes. We also assessed the regional distribution of grooming, directed to the following anatomic areas: forepaws and head, body, hindlegs 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 categorized as being directed to (i) multiple regions or (ii) a single region, and the percentage of grooming bouts and the percentage time spent grooming were calculated for both categories.

To assess nest-building activity, male and female WT and KO mice were housed individually for at least 3 weeks in small plastic cages with standard bedding (aspen chips 4 x 4 x 1 mm, Tapwei Oy, Finland). A standard piece of paper towel (23x23 cm) was provided 3 days prior to inspection, and the nests were assessed by two observers (blind to the genotypes; inter-rater reliability > 0.9), using the following scoring system: 0 – no nests, 1 – primitive flat nests (pad-shaped, consist of a flat paper tissue which slightly elevates a mouse above the bedding), 2 – more complex nests (including warping and biting the paper towel), 3 – complex accurate cup-shaped nests (with shredded paper interwoven to form the walls of the cup), 4 - complex hooded nests, with walls forming a ceiling so the nest becomes a hollow sphere with one opening. To assess maternal nest building, we provided pregnant females (separated from their male partners and kept in big plastic cages for 5-7 days) with a standard

20 x 60-cm paper tissue, and assessed their nest quality on the day 1 postpartum, using the scoring system as described previously.

In addition, blood samples were taken in mice (II), to measure plasma Ca^{2+} levels by atomic absorption spectroscopy (Yhtyneet Laboratoriot, Helsinki, Finland).

Between subjects, each apparatus was thoroughly cleaned (wet and dry cloths). All animal care and experimental procedures in the present study were conducted in accordance with the European legislation and the guidelines of the National Institutes of Health. All animal experiments reported here were approved by the Ethical Committee of the University of Tampere.

8.3. STATISTICS

All results are expressed as mean ± S.E.M. Behavioural data were analyzed by the Mann-Whitney U-test for independent samples (I, III, IV) or by the Kruskal–Wallis test followed by post-hoc U-test (II). A probability of less than 0.05 was considered statistically significant in all tests.

9. RESULTS

9.1. UNALTERED OLFACTION AND VISION (II, III)

Overall, all three genotypes (HZ, WT and KO) produced similar latency of finding the novel object (a 5-cm metal sphere), and the number of contacts with this object (sphere-leaning with forepaws), indicating unaltered sensory (visual) and motor coordination abilities in these mutants (II). In addition, their olfactory abilities (as assessed in the food-finding test) seem to be similar to the WT mice, collectively indicating that major sensory systems, such as olfaction and vision, as well as motor coordination abilities, are unaffected by the VDR gene partial deletion (III).

9.2. INCREASED ANXIETY (I, II, III)

Assessment of general motor activity of the VDR KO mice showed similar baseline activity levels in the KO and WT groups tested in actimeter, including both horizontal and vertical activity (I, II, III). In contrast, marked behavioural difference was found between the genotypes in a battery of anxiety tests, including 1) holeboard test (reduced horizontal and vertical activity), 2) open field (decreased horizontal and vertical exploration, increased grooming duration), 3) elevated plus maze (reduced vertical activity, increased latency to leave the central area), 4) the light-dark transition test (fewer entries to the light compartment, lower vertical activity; I, III).

Assessing the VDR KO mouse horizontal activity in different novelty arena (open field, holeboard) tests, we also noted that these mice differ markedly from their control counterparts in their spatial strategies (Kalueff et al. 2005a). Several behavioural observations have been made. First, we noted that VDR KO mice generally display longer latencies to leave the initial placement area (in the center of the novel arenas, such as holeboard or open field). Similar phenomenon was also observed in the elevated plus maze (I) where the mutant mice demonstrated almost twice longer latency to leave the central zone, compared to their WT or HZ littermates.

Behavioural analyses of leaving the initial placement area also show marked differences between the genotypes. While the controls started to explore unfamiliar arenas after a relatively short freezing episode, and generally did not demonstrate frequent withdrawals (moving backwards) once they "decided" to move, the VDR KO mice showed frequent moving out (2 paws) followed by immediate withdrawals, also constantly changing the vector (direction) of their movements (Fig. 5). In general, these mutants performed 3-9 such withdrawals, before finally leaving the center. Also, as can be seen in Fig. 5, the VDR KO mice (but not their control counterparts) produced frequent stops accompanied by moving forward/backward and vector changes. Overall, all these behavioural observations are consistent with high anxiety phenotype of mice lacking functional VDR.

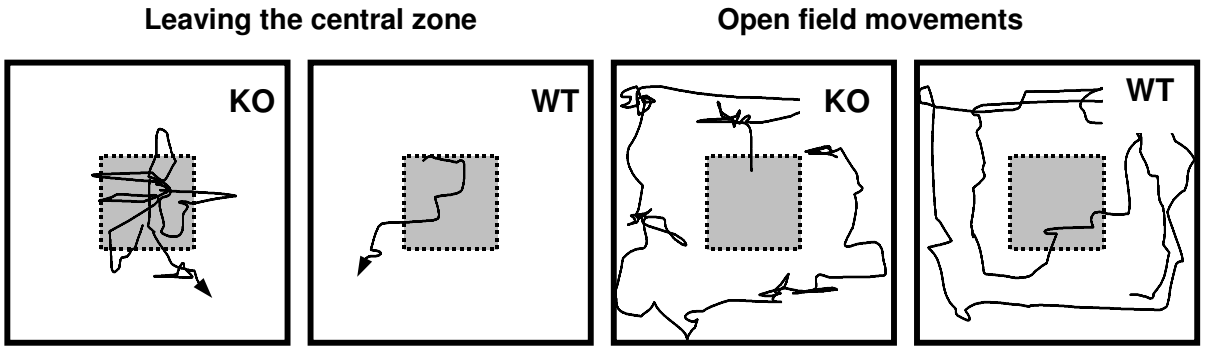


Figure 5. Spatial strategies in VDR knockout (KO) and wild type (WT) mice

9.3. ABERRANT GROOMING ACTIVITY AND PATTERNING (III, IV)

Our experiments show that partial deletion of the VDR gene leads to abnormal grooming in mice, including increased grooming frequency and duration in a battery of tests, such as the actimeter, open field, elevated plus maze and horizontal rod, with a similar trend in the holeboard test (I, III). In addition, we found that VDR KO mice generally display aberrant patterning (sequencing) of their spontaneous (novelty-evoked) or artificial (water-evoked) grooming, including more interrupted bouts, more incorrect transitions, and abnormal regional distribution (more leg grooming, less body and genital grooming, III, IV). Grooming

of HZ mice was similar to the WT group, indicating that these anomalies are inherited as a recessive trait (IV).

9.4. UNALTERED SEXUAL BEHAVIOUR (IV)

In contrast, male mice fed with special rescue Ca/P-rich diet, showed no overt anomalies in their sexual behaviours, as assessed during mating with the estrous WT females in the actimeter test (IV). In fact, they tended to be faster than their WT counterparts in starting sniffing females (7 $\pm$ 2 vs. 14 $\pm$ 4 s, NS), also showing similar number and latencies to follows, attempted mounts and genital self-grooming (IV). Overall patterning (sequencing) of these behaviours was also unaltered in both groups, clearly indicating that sexual behaviour in male VDR KO mice is unaffected by the VDR mutation. In addition, these mice were fertile, and produced viable offspring, if mated with the WT, HZ or VDR KO females.

9.5. CALCIUM LEVELS (II)

Since vitamin D plays an important role in the regulation of Ca^{2+} homeostasis, a key factor likely to affect mouse behaviour may be dysregulation of Ca^{2+} homeostasis, usually associated with vitamin D-related disorders (Carswell 1997; Langub et al. 2001). To test this hypothesis, we measured plasma Ca^{2+} level in VDR KO mice, showing slightly lower plasma Ca^{2+} (2.04 $\pm$ 0.08 mmol/l, $P < 0.05$; n = 6) in this group, compared to the WT (2.28 $\pm$ 0.10 mmol/l, n = 3) and HZ (2.27 $\pm$ 0.48 mmol/l, n = 7) mice (II), whose Ca levels were close to those reported previously to the background 129S1 mouse strain (MPD 2001).

9.6. SPECIFIC MOTOR DEFICITS (II)

In the swim test, the VDR KO displayed robust behavioural anomalies, including abnormal vertical swimming, few immobility, frequent sinking episodes and numerous turns (II). In the vertical screen test, the mutant animals generally showed poorer screen retention time, indicating that the VDR KO group has serious motor defects in their behavioural phenotypes, which are pronounced in tests requiring rigorous activity, such as swimming or screen

retention (II). Interestingly, their horizontal rod balancing performance was unaffected by the mutation (II, III), indicating highly specific nature of these motor disorders.

9.7. IMPAIRED NEST-BUILDING BEHAVIOUR

Overall, the WT males (average nest quality scores: 2.9 $\pm$ 0.1 WT, n = 9, $P < 0.007$, U-test), but not female mice (2 $\pm$ 0.3 WT, n = 5, NS), tended to build more complex, complete and accurate nests, than did their respective VDR KO littermates (1.6 $\pm$ 0.4, n = 7; 1.6 $\pm$ 0.4, n = 7). Figure 6 shows representative nests of the WT and KO mice, including the WT cup-shaped nests with well-assembled walls, compared to the pad-shaped and only slightly wrapped KO nests, consisting of untouched nesting material. The difference between genotypes was unrelated to slower activity levels, since most of nest-building in all groups occurred between days 1-2, indicating that the VDR gene partial deletion impairs nest-building behavioural domain in mice.

9.8. DEFECTS IN MATERNAL BEHAVIOURS

Although VDR KO females mice have long been known to be infertile (Yoshizawa et al. 1997), a high Ca diet has been recently shown to restore fertility in these mice (Johnson and DeLuca 2001). In our study, 100% VDR KO (n = 6) and WT (n = 8) females were able to conceive 2-3 weeks after mating with the HZ males, confirming their fertility and enabling further ethological analyses of the VDR KO maternal phenotypes. We also found significant genotype difference in the litter size, with the VDR KO mice showing the smallest litter size (3.7 $\pm$ 0.33 vs. 5.3 $\pm$ 0.3 WT, $P < 0.01$, U-test).

In addition, we observed a dramatic impairment of the VDR KO maternal behaviour, manifest in poor nest building (predominantly cup-shaped, nest completeness scores: 3.2 $\pm$ 0.2 vs. more complex hooded nests, 4 $\pm$ 0 WT, $P < 0.006$), abnormal mothering, and cannibalism. While pup-killing was absent in the WT females (0%, $P < 0.01$, U-test), 100% of pups born by the VDR KO mice were killed by their mothers 1-5 days after birth. They also neglected their pups, often leaving them unattended and resting outside the nest, as assessed during our homecage observations (data not shown). These impairments were not

observed in the WT group, displaying extensive nests and normal maternal behaviours, including regular grooming and arched-back nursing of their pups, typical for background 129S1 mouse strain, known for its good mothering style (MPD 2001).

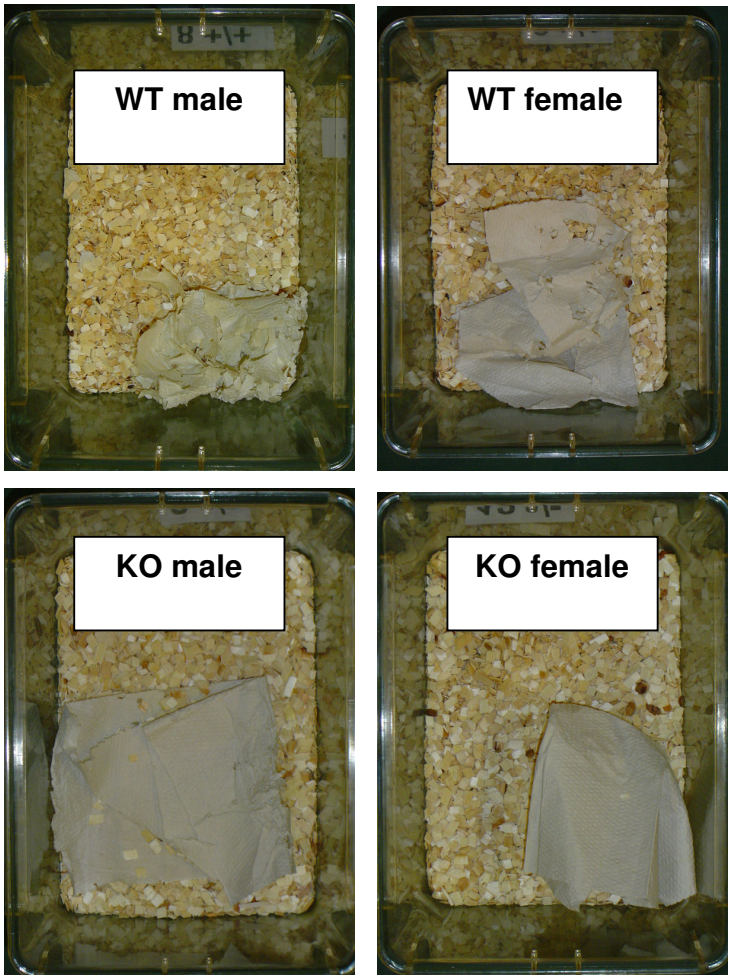


Figure 6. Impaired nest-building behaviour in the VDR knockout (KO), compared to the wild type (WT) mice. Photographs show examples of nests built 3 days after introduction of nesting material (paper towel) into the cage

9.9. CORRIGENDUM

Due to oversight of records and incorrect sexing of several animals, the age/sex of animals were slightly incorrectly indicated in the original communications (6-8 month instead of 7-15, mean: 12 months). The WT group consisted of 11 mice, and the KO group consisted of 6 males and 4 female mice. This discrepancy, however, did not affect the results of the present study, since the age belonged to the same adult-to-aged group (according to the standard phenotyping protocols, Crawley 1999, 2000), and there was no difference between male and female VDR KO mouse behaviours (Fig. 7), with similar behavioural profiles, as assessed in several additional post-hoc experiments, performed in both male and female mice (Table 3).

Table 3. Summary of behavioural profiles of female VDR knockout mice (n = 10) tested in a battery of activity and anxiety tests

Test	Behavioural profile (vs. the wild type group)	Similarity to male VDR KO phenotype (I-IV)
Actimeter test	Unaltered activity scores	+
The light-dark test	Reduced exploration (light entries + looks)	+
Open field test	Reduced horizontal and vertical exploration	+
Holeboard test	Reduced horizontal and vertical exploration	+
Elevated plus maze	Trend to reduced head dips	+
Novelty-induced grooming test	Aberrant sequencing (interruptions) and regional distribution (more rostral, less caudal grooming)	+
Swimming	Impaired (more sinking and turns)	+

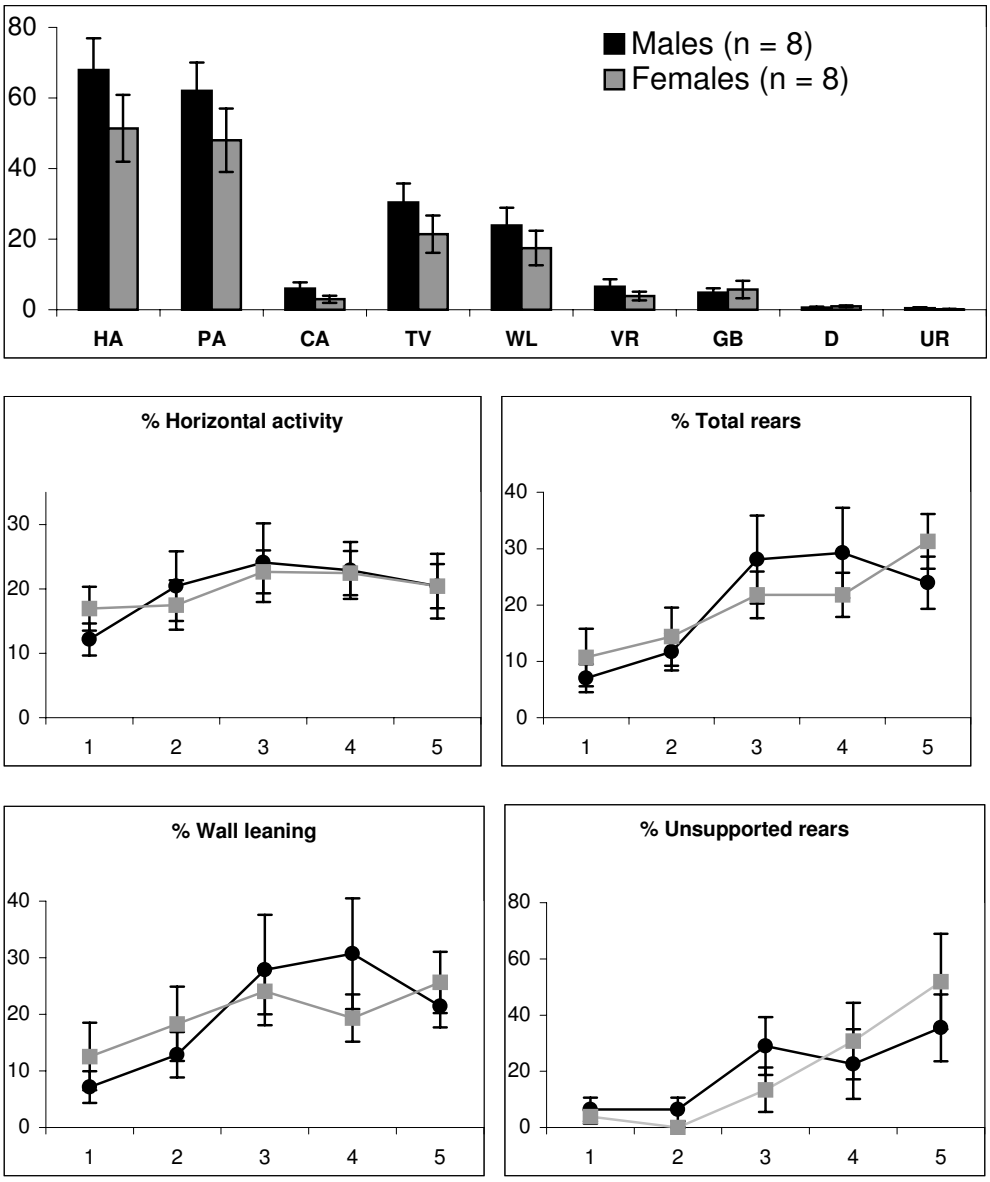


Figure 7. Comparison between male and female VDR knockout mice in the 5-min the open field test (gross scores and temporal per-minute distribution of activity, % of total). HA – total, PA – peripheral, CA – central horizontal activity, TV – total vertical activity, WL – wall leaning, VR – unsupported vertical rears, GB – grooming bouts, D – defecation boli, UR – urinations

10. DISCUSSION

Creation of the VDR KO (Yoshizawa et al. 1997; Kato et al. 1999) had provided new insights into the role of VDR in different tissues (including brain), and enabled studies of the role of the vitamin D/VDR system in the regulation of behaviour. Our previously published works (I-III) were the first reports characterizing behavioural phenotypes of these mice. Recently, some additional behavioural anomalies have been reported for these mice (Burne et al. 2005), emphasizing the growing interest in the use of the VDR genetic ablation as an animal model of human vitamin D-related brain disorders (Table 4).

10.1. ANXIETY PHENOTYPE

Overall, our data show consistent reduction of horizontal and vertical exploration in our VDR KO mice, indicative of their high anxiety phenotype. Several additional behavioural phenomena seem to support our hypothesis linking non-functional VDR to anxiety (I). First, although not statistically significant, lower head dipping scores have been reported for VDR KO mice in the elevated plus maze by Burne et al (2005). Second, since the marble burying test represents a traditional exploratory-based anxiety model (Londei et al. 1998), lower burying activity in the VDR mutant group (Burne et al. 2005) may also indicate higher anxiety in these mice compared to their WT counterparts. Importantly, our findings (I) are consistent with data from the literature showing that rodent stress and anxiety are generally associated with decreased exploration (Crawley 1999, 2000; Prut and Belzung 2003). Furthermore, since alterations in vertical activity reflect emotional components of rodent behaviour (Prut and Belzung 2003), our results are consistent with earlier studies in rats (Althemus et al. 1987) linking vitamin D deficiency to inhibition of open field vertical activity. In line with this, the highest brain VDR concentration has been found in the limbic system, the key emotiogenic brain structure, and its extensions in the brain (Walbert et al. 2001; Langub et al. 2001).

Table 4. Comparison of the available published studies characterizing behaviours of Tokyo VDR knockout male mice

Behaviours and tests	Studies	
	I, II, III, IV	**Burne et al. 2005**
Vertical exploration		
open field	(-)	(-)
elevated plus maze	(-)	(-)
light-dark box	(-)	N/a
holeboard	(-)	(-)
Horizontal activity		
open field	Trend: (-)	(-)
elevated plus maze	0	(-)
light-dark box	0	N/a
holeboard	(-)	(-)
Y-maze	N/a	(-)
Additional exploratory behaviours		
hole-poking (holeboard)	0	Trend: (-)
head-dips (plus maze)	N/a	0
open time (plus maze)	N/a	0
Y-maze arm alternation	N/a	(-)
Other anxiety-sensitive behaviours		
urination	0*	(-)**
defecation	0 ***	0**
acoustic startle response	N/a	(-)
marble burying	N/a	(-)
Other behaviours		
memory in Y-maze	N/a	0

digging (bedding test)	N/a	(-)
open field habituation ****	N/a	(-)
acoustic response habituation	N/a	(-)
self-grooming ***	(+)	0 *****
pre-pulse inhibition ******	N/a	(-)

N/a – not assessed. (+) – activation, (-) - reduction, 0 – no effects. * Unpublished data (I). ** Open field data. *** In a battery of 4 tests. **** Horizontal activity (distance traveled). ***** Holeboard test data. ****** To acoustic startle response (at long 256 ms, but not short 8-64 ms intervals).

Importantly, 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 (Garcion et al. 2002; Carswell 1997; Gloth et al. 2001).

10.2. POSSIBLE CONFOUNDING FACTORS

Robust light-dark behavioural shifts, commonly seen in anxious mice in the light-dark test (Crawley 2000), were absent in the VDR KO mice (I), suggesting that this paradigms may not be suitable for these mice (perhaps, due to high test aversion and/or highly anxious hypoactive 129S1 background; Crawley 1999, 2000). Likewise, relatively poor "anxiogenic" performance of these mice in the holeboard and elevated plus maze tests, including specific nose-poking and open:closed behaviours (I, III, Burne et al. 2005) may be confounded by alopecia – a common diet-resistant phenotypic feature of all mice lacking functional VDR (Li et al. 1997; Yoshizawa et al. 1997). Indeed, VDR KO mice have little body hair and few whiskers by the age of 3 months (Fig. 4), whereas both HZ and WT groups have normal whiskers even at the age of 1-1.5 years. Tactile information obtained from whiskers is an important sensory input guiding novelty exploration behaviour in rodents (Prchal et al. 2004). Since the whisker status of mice has been reported to affect their performance in the elevated plus maze, leading to unusually low aversion of the open arms (due to sensory impairment and inability to detect potential danger in the open arms; see Crawley 1999 for details), the lack of whiskers in VDR mutant animals may be the main reason for their "unusual"

responses in the elevated plus maze. Thus, the elevated plus maze may represent an inappropriate model to study anxiety in these VDR KO mice.

Similar conclusion may apply to the holeboard test – another highly sensitive behavioural paradigm widely used in behavioural neurogenetics (Crawley 1999; Kalueff and Tuohimaa 2004b). Both groups studying VDR mutant mice (I, Burne et al. 2005) failed to detect any alteration in the specific anxiety-sensitive exploratory activity - nose poking. However, we shall note the important role of whiskers in nose-poking behaviours in rodents, and the fact that VDR mutant mice (but not their WT counterparts) have no whiskers. Clearly, animals without whiskers will differ in the holeboard test, demonstrating altered nose-poking activity. Taken together, this suggests that the "lack" of anxiety response of VDR mutant mice tested in the holeboard test may be due to poor ability of this model to detect anxiety in these animals.

In addition, 129S1 genetic background, known to be insensitive to the open:closed stimuli in the elevated plus maze, may also contribute to these finding, further demonstrating how different interplaying factors may determine complex behavioural phenotypes observed in the VDR KO mice (Kalueff et al. 2005a). From this point of view, it may also be interesting to assess behavioural phenotypes in VDR KO mice on other genetic backgrounds, markedly different behaviourally from the 129S1 strain used here (Kalueff et al. 2004c; Kalueff and Tuohimaa 2004a, 2005b). We are currently transferring the VDR null mutation on several new genetic backgrounds (C57, BALB and NMRI) in order to perform such comparative studies. Moreover, it may also be important to examine the behavioural profiles of mutant mice with other abnormalities in the vitamin D system. For example, mice lacking 25-dihydroxyvitamin D-1α-hydroxylase, a key enzyme of vitamin D bioactivation (Fig. 2), are currently available for biomedical research (St-Arnaud 1999a; Kato 2001; Kato et al. 2002). Displaying phenotypes resembling that of the VDR KO, these mice may be a useful tool to further dissect the role of the vitamin D/VDR system in the regulation of behaviour. In addition, mice deficient in the 25-hydroxyvitamin D-24-hydroxylase enzyme, and showing impaired 1,25-D metabolism (St-Arnaud 1999b, St-Arnaud et al. 2000) may also be a useful tool for such studies.

10.3. GROOMING PHENOTYPE

Altered grooming phenotype was a strikingly consistent finding in our VDR KO mice (I, III, IV). Explaining these data, we note that grooming is a particularly important part of the rodent behavioural repertoire, sensitive to a number of endogenous and exogenous manipulations, and representing a complex hierarchically organized behaviour, with a general pattern of cephalocaudal progression (paw licking, nose and face wash, head wash, body wash and fur licking, leg licking, tail/genitals licking and wash) (Berridge et al. 1987; Sachs 1988; Berridge 1989; Spruijt et al. 1992; Berridge and Whishaw 1992; van Erp et al. 1994; Aldridge and Berridge 1998; Kruk et al. 1998; Berridge and Aldridge 2000; Greer and Capecci 2003; Kalueff and Tuohimaa 2004a).

Several data may link the vitamin D system to grooming. First of all, grooming itself (in addition to coprophagy) may be an important source of vitamin D, obtainable from UV-irradiated body grease (Carpenter and Zhao 1999). Therefore, it is tempting to speculate that, at the level of behavioural adaptations, grooming activity of animals may be evolutionary linked to the overall vitamin D status. In other words, where vitamin D-VDR signalling seems to be impaired, indicating “low vitamin D” status (e.g., hypovitaminosis D, VDR ablation), animals might try to “compensate” this dysfunction by increasing their grooming. Second, high concentrations of VDR were 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 and Norgen 1978; Kruk et al. 1998; Strazielle and Lalonde 1998). Finally, vitamin D is involved in VDR-mediated modulation of brain neurotransmitters, including acetylcholine and dopamine, both known to regulate grooming (Carswell 1997; Cromwell et al. 1998; Garcion et al. 2003). Given these data and our own results (III, IV), it is possible to suggest that the vitamin D system and VDR may be important for the regulation of grooming and its sequencing, and that partial deletion of the VDR gene dramatically affects this behavioural domain.

10.4. OTHER BEHAVIOURAL DOMAINS

Since early reports indicated possible role of the vitamin D system in the regulation of sexual behaviour (Mirzahosseini et al. 1996), this behavioural domain was also interesting to assess in this study. However, both the KO and control mice showed unaltered sexual behaviour and its patterning (IV), suggesting that this behavioural domain is not affected by VDR null mutation, as long as Ca metabolism is corrected in these animals.

Analysing several other behavioural domains, we note robust genotype differences in nest-building and maternal behaviours, suggesting impairment of these domains by VDR genetic interruption. Notably, numerous data show the important role of VDR in the regulation of prolactin gene expression in different tissues (Delvin et al. 1990; Castillo et al. 1999), suggesting that the vitamin D/VDR and prolactin endocrine systems may interact (Robinson et al. 1982). It is possible to suggest that such interaction is impaired in VDR KO mice, leading to their abnormal prolactin-dependent mechanisms. Given the key role of prolactin in the regulation of nest-building and maternal behaviours in mice (Voci and Carlson 1973), this hypothesis, if true, may explain both abnormal maternal and nest-building behaviours, observed in the VDR KO mice in the present study. Moreover, co-localization of VDR with oxytocin in hypothalamic neurons (Prufer and Jirikowski 1997) implies some degree of interplay between the oxytocin and vitamin D endocrine systems. Given the key role of oxytocin in maternal behaviour (Ragnauth et al. 2005), and possible impairment of oxytocin-VDR interplay in mice lacking functional VDR, this may also contribute to disturbed maternal behaviours observed here in VDR KO mice.

10.5. CALCIUM AND MOTOR/SENSORY SYSTEMS

Since the assessment of general physiological status, vestibular, sensory and locomotor systems is a key part of behavioural phenotyping of mutant animals (Crawley et al. 1999), these factors had to be considered in our VDR KO mice. VDR deficiency in knockout mice has recently been reported to yield aberrant musculoskeletal development as well as rickets-like bone malformations (Yoshizawa et al. 1997; Endo et al. 2003). However, using different

behavioural tests we showed that VDR KO mice, receiving special rescue diet, exhibited unaltered baseline motor activity, movements coordination and sensory abilities (I-IV). In addition, special diet used in our study (known to normalize all major physiological functions and reduce or eliminate muscular abnormalities in VDR mutants) also led to sufficient normalization of Ca plasma levels in mice tested in our behavioural experiments (II). In contrast, Burne et al. (2005) found aberrant motor functions in VDR KO mice, including poor rotarod performance and a shorter stride length, suggesting that these mice may have severely impaired muscular and motor functions rather than anxiety phenotype. We shall note, however, one principally important methodological difference between the two studies. While we used special Ca/Ph-rich diet with 20% lactose (II), Burne et al. (2005) used water supplemented with 2mM Ca^{2+}. Since lactose markedly increases Ca absorption from food, we may suggest that our diet was more effective to normalize mineral homeostasis in VDR mutant mice. Indeed, while our KO mice showed only moderate 11% hypocalcemia (2.04 vs. 2.28 mmol/l in controls), the mice used by Burne et al. (2005) displayed markedly lower plasma Ca (1.7 vs. 2.5 mmol/l in controls). In our opinion, this 0.8-mmol/l difference in Ca levels between both genotypes may be considered as severe hypocalcemia (32%), representing a serious factor non-specifically impeding all behaviours in VDR KO mice. Therefore, motor impairments seen by Burne et al. (2005) may indeed be due to Ca-dependent imbalance in these mice, possibly due to insufficient Ca supplementation.

However, these data to not contradict our findings. Indeed, the difference in plasma Ca between genotypes observed in our experiments (0.24 mmol/l) was almost 3-fold lower than in (Burne et al. 2005). We may suggest that this mild hypocalcemia may not lead to dramatic behavioural impairments in the VDR KO group (I-IV), therefore enabling more specific assessment of behavioural phenotypes of these mutant mice. Notably, both cited studies support the notion that mineral homeostasis affected by VDR null mutation may strongly affect animal behavioural performance, and therefore has to be carefully controlled in VDR KO mice.

10.6. PATTERN OF INHERITANCE

Analyzing behavioural phenotypes in WT, HZ and KO mice, we noted that impaired behaviours were only seen in VDR KO animals, remaining unaltered in both HZ and WT groups (I, IV). This observation is in line with our data (II) showing almost identical behavioural phenotypes of HZ and WT mice in several anxiety and motor tests. In line with this, numerous observations show that HZ mice display similar VDR gene mRNA expression, have no overt anomalies, and phenotypically are indistinguishable from their WT littermates (Yoshizawa et al. 1997; Kato et al. 1999). Moreover, all other mutant mice with non-functional VDR (e.g., Li et al. 1997; Erben et al. 2002) also reveal similar phenotypes in HZ and WT animals. Several conclusions can be made based on our present findings. First, similar "normal" behavioural and physiological phenotypes of HZ and WT mice implies that only one WT control group is necessary for behavioural analysis of VDR KO mice. Second, our results reveal the degree of dominance (i.e. harmful recessive) observed for behavioural phenotypes of these mutant mice. Third, given recent data on reduced VDR level in HZ mice (Yoshizawa et al. 1997; Kallay et al. 2001), our findings raise the possibility that a limited number of functional VDR may be enough to maintain both normal physiological and behavioural 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 HZ carriers (Malloy et al. 1997). Taken together, these findings support the notion that that physiological and behavioural anomalies induced by VDR impairment are inherited as a recessive trait in both mice and humans.

10.7. POSSIBLE ADDITIONAL MECHANISMS

Several other potential mechanisms, underlying neuroactive effects of 1,25-D, may be suggested to contribute to our findings. For example, it is possible to assume that vitamin D modulates the brain neuromediators and receptors, see (Carswell 1997; Garcion et al. 2002) for details. Notably, since gamma-amino butyric acid GABA-A receptors represent an important target for non-genomic action of many neurosteroids and neuroactive hormones (Losel and Wehling, 2003; Losel et al. 2003), it is tempting to speculate that 1,25-D may act

in the brain in a similar way, modulating neuronal excitability and other neurophysiological phenomena. However, our data show the importance of functional VDR for normal brain functioning, thus, negating this direct link in our results.

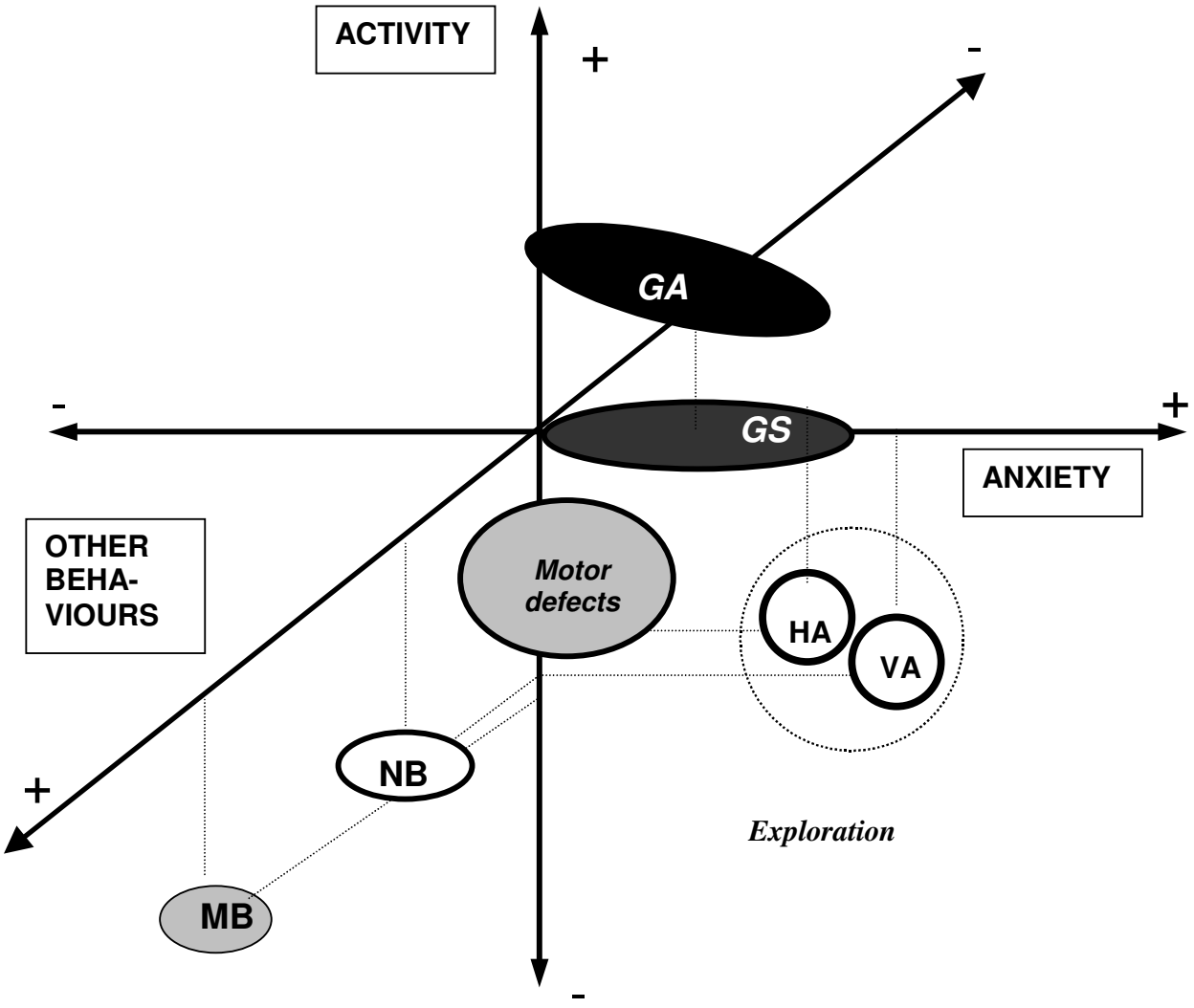


Figure 8. Schematic presentation of behavioural domains affected in Tokyo VDR knockout mice. GA – grooming activity (frequency, duration: increased), GS – grooming sequencing (behavioural microstructure: impaired), HA – horizontal and VA – vertical activity (reduced), NB – nest-building (impaired), MB – maternal behaviours (impaired)

Nevertheless, vitamin D depletion has been recently reported to reduce the expression of α4, and slightly down-regulate α1, subunits of GABA-A receptors (Feron et al. 2005). Thus, the lack of vitamin D-VDR signaling in our VDR KO mice may lead to similar effects, also affecting these subunits in the brain. Given the crucial role of GABAergic system in the regulation of behaviours, especially anxiety and grooming (Kalueff and Nutt 1996; Kalueff and Tuohimaa 2005a), this possibility seems indeed likely, justifying further studies in our VDR KO mice. If true, this hypothesis might explain increased anxiety and aberrant grooming phenotype observed in this study (I, IV).

Interestingly, co-localization of VDR with calbindins in the brain suggests their potential interaction (Sutherland et al. 1992). In VDR-ablated mice, calbindin-D9k (but not calbindin-D28k) mRNA was dramatically reduced in the brain, further strengthening the link between VDR and calcium-binding proteins in the CNS (Li et al. 1998b). Since Ca-binding proteins are important for brain functions, and their imbalance results in marked behavioural and sensory deficits in mice (Airaksinen et al. 1997; Schwaller et al. 2002; Barskii et al. 2003), it is possible that partial deletion of the VDR gene alters the expression of brain endogenous Ca-binding proteins, thus indirectly contributing to our findings in VDR KO mice.

Clearly, several other neurotransmitter systems, as well as brain cytokines and other key proteins may also be involved, as summarized in Table 5, and require further in-depth studies in these mutant mice. The extend of behavioural anomalies observed in this study, differentially affecting several important behavioural domains, suggests that multiple pathogenic mechanisms may be involved, underlying our results (I-IV) and data coming from other groups (Burne et al. 2005).

10.8. GENERAL SUMMARY

In summary, our study shows that VDR KO mice display higher anxiety and abnormal grooming phenotypes, as well as impaired swimming, nest-building and maternal behaviours (Fig. 8). A striking similarity of our findings, describing behavioural phenotypes of VDR KO mice (I-IV), with recently published data of another group (Burne et al. 2005, Table 4) confirms overall generality of our behavioural observations.

Table 5. Summary of possible targets of Vitamin D in the brain

- **VDR-related mechanisms**
    - Brain development and differentiation
    - Neuroprotection
    - Neurotrophins and cytokine release
    - Expression of neurotransmitter metabolism enzymes
    - Modulation of peptidergic systems
    - Expression of Ca channels and Ca-binding proteins
- **VDR-unrelated mechanisms**
    - VDRm ?
    - Direct steroid-like effects:
        - Membrane effects ?
        - Neurosteroid-like modulation of neurotransmitter receptors?
- **Secondary (indirect) mechanisms**
    - Altered Ca/P metabolism
    - Renin-angiotensin system, hypertension

In general, our data give further support to the crucial role of vitamin D in the brain, and contribute to the growing recognition of the importance of functional VDR in the regulation of behaviour (Carswell 1997; Garcion et al. 2002; Burne et al. 2005; I-IV), Table 5. Given accumulating clinical data linking various VDR mutations to a variety of psychiatric phenotypes (Ozer et al. 2004; Tajouri et al. 2005; Yan et al. 2005), VDR KO mice may emerge as a useful animal model to study different brain disorders. In addition, we can also hypothesize that new psychotropic drugs can be created based on targeting the vitamin D/DVR neuroendocrine system.

11. CONCLUSIONS

1. This work was the first study behaviourally characterizing mutant mice lacking functional nuclear VDR, and demonstrating that these mutants display a number of behavioural anomalies, including several distinct behavioural domains.
2. In a battery of anxiety and activity tests, VDR KO mice generally display increased anxiety phenotype, manifest in stress-evoked reduction of their exploration, especially vertical activity (rearing).
3. Increased self-grooming activity, consistently seen in various behavioural tests, represents another behavioural feature of these mice.
4. Behavioural patterning of grooming is dramatically affected in these mutant mice, manifest in increased interrupted bouts, aberrant sequencing and abnormal regional distribution of grooming activity.
5. Partial deletion of the VDR gene results in a marked, but highly specific motor, impairments in these mice, affecting their performance in tests requiring rigorous physical activity, such as the vertical screen retention (poorer retention) and the swim tests (inability to swim).
6. Sexual behaviours as well as olfaction, vision and motor coordination appear to be unaffected by the mutation in these mice.
7. VDR KO mice display aberrant nest-building and impaired maternal behaviours, including pup neglect and 100% cannibalism.
8. Overall, our results confirm the important role for the vitamin D/VDR neuroendocrine system in the regulation of behaviour, and contribute to our understanding of its physiological roles in the brain.

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After all, if there is anything still left unknown about VDR knockout mouse behaviour – it is thanks to the family and friends, keeping the dissertant away from the lab. This gap, however, will be easily filled – and some future dissertations will cover this fascinating topic one day.

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Allan V. Kalueff

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14. ORIGINAL COMMUNICATIONS