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Vitamin D and the Nervous System

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the Small Auditorium of Building B,
Medical School of the University of Tampere,
Medisiinarinkatu 3, Tampere, on December 22nd, 2008, at 12 o'clock.

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
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Tampere Graduate School in Biomedicine and Biotechnology (TGSBB)
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Cover design by
Juha Siro

Acta Universitatis Tamperensis 1372
ISBN 978-951-44-7546-7 (print)
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 794
ISBN 978-951-44-7547-4 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2008

To my family

“FORTUNE FAVORS THE BOLD”

CONTENTS

1 ABSTRACT

Vitamin D_3 is a fat-soluble seco-steroid hormone, which is synthesized in the skin (under the influence of ultraviolet light from the sun) or obtained from food. Metabolites of vitamin D_3, 25-hydroxyvitamin D_3 (25(OH)D) and 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$), have their effects through the vitamin D receptor (VDR), and alter the expression of specific target genes. The vitamin D_3 endocrine system is essential for calcium and bone homeostasis. Many brain cells, several regions of the central nervous system and muscle cells express VDRs. The central nervous system is increasingly recognized as a target organ for vitamin D_3. Several clinical and experimental/animal data show regulation of physiological brain functions (such as neuroprotection, antiepileptic effects, immunomodulation, regulation of behavior, as well as motor functions) by vitamin D. The present study was focused on the biological action of the vitamin D-VDR system in adult nervous system, as revealed by studying animals with genetically modified VDR, or genes involved in vitamin D metabolism. VDR knockout mice develop abnormal emotional behaviors, such as neophobia, and increased seizures following pentylenetetrazole administration. $1,25(OH)_2D$ also regulates seizure activity in two wild type mouse strains. The gustatory and olfactory functions, spatial memory, hedonic responses of VDR deficient mice were unimpaired, although the mutants displayed impaired hearing and balance functions. VDR mutant mice of two different strains also developed swimming impairments. Collectively, the present study demonstrates that vitamin D regulates various functions of the nervous system, confirming that the brain could be considered as a target organ for vitamin D.

2 ABBREVIATIONS

AD	Alzheimer's disease
AF	Activating factor
BDNF	Brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
CaBP	vitamin D dependent calcium binding protein
CYP	microsomal cytochrome P_{450}
CYP24	25-hydroxyvitamin D-24-hydroxylase
CYP27B1	1 α-hydroxylase or the cytochrome P_{450} enzyme responsible for 25-hydroxyvitamin D-1 α-hydroxylation, or 1α-OH
DBD	DNA- binding domain
DBP	vitamin D binding protein
DC	dendritic cell
DVD	vitamin D deficient
EcaC1, EcaC2, CaT1	epithelial calcium channels
FGF23	phosphaturic factor, fibroblast growth factor 23
GABA	gamma aminobutyric acid
GDNF	glial cell derived neurotrophic factor
γ-GT	gamma-glutamyl transpeptidase
GTH	glutathione
^3H	tritium labeled
HPA	hypothalamic pituitary axis
Hsp	heat shock proteins
HVDRR	hereditary vitamin D resistant rickets
HZ	heterozygous
IDPB	Intracellular D binding proteins

IHC	immunohistochemistry
iNOS	inducible nitric oxide synthase
LBD	ligand -binding domain
L-VSCCs	L-type voltage-sensitive Ca++ channels
memVDR	membrane vitamin D receptor
MS	multiple sclerosis
NCX	Na+/Ca++ exchanger
NGF	nerve growth factor
NR	nuclear receptor
NT	neurotrophins
PDDR	pseudo vitamin D deficiency rickets
PKC	Protein kinase C
PMCA1	basolateral plasma membrane ATPase
PTH	parathyroid hormone
PTZ	pentylenetetrazol
ROS	reactive oxygen species
RXR	retinoid X receptor
TRPV	transient receptor potential vanilloid channel family, TRPV5 and TRPV6
UVB	ultraviolet B
VD	vitamin D_3 or vitamin D_2
VDR	vitamin D receptor
VDDR-I	Vitamin D-dependent rickets type I or pseudovitamin D-deficiency rickets type I
VDDR-II	Vitamin D-dependent rickets type II
VDRE	vitamin D response element
VDR KO mice	VDR knockout mice
WT	wild type

$1,25(OH)_2D$	1,25-dihydroxyvitamin D_3, calcitriol or 1,25-dihydroxyvitamin D_2
1 α-OH KO mice	1 alpha-hydroxylase knockout mice
25(OH)D	25-hydroxyvitamin D_3 or 25-hydroxyvitamin D_2, calcidiol
7DHC	7-dehydrocholesterol

3 LIST OF ORIGINAL COMMUNICATIONS

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

I. Kalueff AV, Minasyan A, Tuohimaa P (2005): Anticonvulsant effects of 1, 25-dihydroxyvitamin D in chemically induced seizures in mice. Brain Res Bull. 67(1-2):156-60.

II. Kalueff AV, Minasyan A, Keisala T, Kuuslahti M, Miettinen S, Tuohimaa P (2006) Increased severity of chemically induced seizures in mice with partially deleted Vitamin D receptor gene. Neurosci Lett. 394(1):69-73.

III. Jing Zou, Anna Minasyan, Tiina Keisala, Ya Zhang, Jing-Huan Wang, Yan-Ru Lou, Alan Kalueff, Ilmari Pyykkö and Pentti Tuohimaa (2008): Progressive Hearing Loss in Mice with a Mutated Vitamin D Receptor Gene. Audiol Neurootol. 13(4):219-230 (Shared paper with MSc Tiina Keisala).

IV. Minasyan A, Keisala T, Lou YR, Kalueff AV, Tuohimaa P (2007): Neophobia, sensory and cognitive functions, and hedonic responses in vitamin D receptor mutant mice. J Steroid Biochem Mol Biol. 104(3-5):274-80.

V. Minasyan A, Keisala T, Zou J, Ya Zhang, Toppila E, Syvälä H, Lou YR, Kalueff A, Pyykkö I, Tuohimaa P (2008): Balance deficits in vitamin D receptor mutant mice. (Submitted to J Steroid Biochem Mol Biol).

4 INTRODUCTION

The hormonally active form of vitamin D (VD), 1α,25-dihydroxyvitamin D ($1,25(OH)_2D$) is formed by hydroxylation of 25-hydroxyvitamin D - 25(OH)D - and its physiological functions include a classical role in calcium homeostasis and bone metabolism. However, several recent studies have postulated functions for $1,25(OH)_2D$ during the CNS development, and in the adult brain. Specifically, $1,25(OH)_2D$ modulates the activity of key brain genes and enzymes of neurotransmitter metabolism, and is also involved in autoimmune responses and neuroprotection.

Both animal and clinical studies strongly support the notion that chronic VD deficiency is harmful to brain development and to adult neural functions. For example, in rodent models, VD deficiency leads to brain malformation and has effects on rodent behavior. In humans, it is associated with mood disorders, multiple sclerosis, schizophrenia, and epilepsy. The functions of VD are mediated through the nuclear VD receptor (VDR), a member of the nuclear receptors (NR). VDR is widespread in both the developing and adult brain, as well as in the spinal cord, suggesting a potential role for VD and VDR in the brain.

Targeted deletion of genes encoding 25(OH)D 1α-hydroxylase (CYP27B1, 1α-OH) and of nuclear VDR have provided useful mouse models of inherited human diseases such as VD-dependent rickets type I and II (VDDR-I and VDDR-II). The absence of functional CYP27B1 and VDR creates a bone and growth plate phenotype, which are clinical features of rickets, due to the absence of the active VD metabolite, $1,25(OH)_2D$, and the unresponsiveness of organs to VD. The intestine is one of the target organs for VD/VDR, as high calcium intake restores normal bone and growth plate phenotypes.

These mutant mice are a valuable tool to assess the role of VD in the regulation of the brain functions. Insufficient VD levels or genetic deletion of genes that are required for synthesis of VD/VDR, have been already reported in animals and humans to lead to the increase of risk of several neurological diseases. However, it is not completely clear whether VDR is involved in the regulation of specific functions of the CNS, such as behavior or seizure activity.

This thesis aimed to study some neurophenotypes of VDR knockout (KO) mice, as well as in 1 alpha-hydroxylase knockout mice (1α-OH KO). The precise mode of action and the full spectrum of activities of the VD hormone can be better understood based on using these mice.

5 REVIEW OF THE LITERATURE

5.1 VITAMIN D

5.1.1 History of vitamin D

In the 17th century, the first clinical picture of rickets was reported by a Dutch physician, who described the thoracic malformation in rickets (Ebstein, 1908; Tausk, 1649) . The origin of the disease was unknown until the discovery in the beginning of the 20th century of a nutritional compound from cod liver oil that prevented rickets (McCollum et al., 1922; Mellanby, 1919). The first scientific credence for VD was given by Sir Edward Mellandby in Great Britain, where he explained the dietary importance of VD in preventing rickets (Mellanby, 1919). Mellanby discovered from experiments, assisted through McCollum who provided ricketic dogs, that cod liver oil cured the disease. Later, McCollum found that the healing of rickets was due to a new substance which he termed “Vitamin D” (Mellanby, 1919). At the same time, it was shown that rickets in children could be cured or prevented by exposure to sunlight (Chick, Palzell, and Hume, 1923; Hess, 1922; Huldshinsky, 1919). Scientists realized that two factors were essential for healing rickets: exposition to sun and a diet rich in VD. The chemical identification and synthesis of VD brought A. Windaus a Nobel Prize in chemistry in 1938 (Windaus and Linsert, 1928). Two chemical forms of VD are presented in Fig 1 - vitamin D_2 (ergocalciferol) and D_3 (cholecalceferol), which are known as steroid prohormones. VD_2 was isolated through the irradiation of plant sterols (Windaus and Linsert, 1928), whilst VD_3 is synthesized in the skin of vertebrates (Windaus, Lettre, and Schenck, 1935).

Another major step in the history of VD was the discovery of VD metabolites, which are biologically more active (Lund and DeLuca, 1966). The 25(OH)D calcidiol (Blunt and DeLuca, 1969; Blunt, DeLuca, and Schnoes, 1968) and 1,25$(OH)_2$D calcitriol (Holick et al., 1971; Lawson, Wilson, and Kodicek, 1969), were isolated, chemically identified and synthesized. Although they have different antirachitic activities (Eliot, 1938), calcitriol is generally accepted as the primary hormonally active form of VD, as reviewed in (Bikle, 1992; Bikle and Pillai, 1993; Bouillon, Okamura, and Norman, 1995; Brommage and DeLuca, 1985; DeLuca, 2004; Fraser, 1980; Holick, 2007).

5.1.2 Metabolism of vitamin D

VD is a fat-soluble seco-steroid hormone. Humans and mammals acquire VD through their diet and from exposure to sunlight. A diet high in oily fish prevents VD deficiency. During exposure to sunlight, ultraviolet B (UVB) radiation (290 - 315 nm wavelength) is absorbed by 7-dehydrocholesterol, a cholesterol-like molecule (7DHC), and it forms pre-VD (Cannell and Hollis, 2008; Cantorna et al., 2004; DeLuca, 2004; Dusso, Brown, and Slatopolsky, 2005; Holick, 2006; Holick, 2007; Zittermann, 2003). This is a purely photochemical reaction where the enzymes do not participate. Any excess of pre-VD or VD is destroyed by sunlight and does not cause VD intoxication, however, increased melanin pigmentation reduces the efficiency of UV (Holick, 1981; Holick et al., 2007; Zittermann, 2003). Orally ingested and endogenously formed VD is inert and does not have any significant biological activity before it is metabolized within the body into the active hormonal form. This occurs in two steps within the liver and kidneys (DeLuca, 2004; Dusso, Brown, and Slatopolsky,

2005; Holick, 2003; Lips, 2006). In the liver, VD is metabolized to 25(OH)D (Fig 1). Several hepatic microsomal cytochrome P-450s (CYP) have been shown to hydroxylate, but CYP2R1 is most likely the key enzyme (Cheng et al., 2004; Cheng et al., 2003). Patients with classical rickets, due to homozygous mutations, have low circulating 25(OH)D levels (Cheng et al., 2004).

The regulation of these 25-hydroxylases is not clear, but serum 25(OH)D reflects the nutritional status, and is used to determine a patient's VD status. The regulation of this step is weak and there is no significant storage of 25(OH)D in the liver of mammals (Cannell and Hollis, 2008; Dusso, Brown, and Slatopolsky, 2005; Zittermann, 2003), however the intake of VD enhances the level of 25(OH)D in plasma (Holick, 1981). 25(OH)D is rapidly released by the liver into blood and has a half-life in circulation of two weeks(Holick, 1981). Unlike hepatic 25-hydroxylases, it appears only one 25(OH)D-1α-hydroxylase (CYP27B1), which is expressed at highest concentration in the kidney, hydroxylases at 1α position renal synthesis of $1,25(OH)_2D$ is stimulated by plasma parathyroid hormone levels and decreased by serum calcium and phosphorus levels (DeLuca, 2004; Dusso, Brown, and Slatopolsky, 2005; Lips, 2006). Fibrolast growth factor 23, secreted from the bone, also suppresses $1,25(OH)_2D$ synthesis (Holick, 2007). In cells treated with $1,25(OH)_2D$, reduced activity of CYP27B1 mRNA was seen (Monkawa et al., 1997; Shinki et al., 1997). Another factor, klotho gene, acts as a negative control of CYP27B1 (Tsujikawa et al., 2003; Yoshida, Fujimori, and Nabeshima, 2002) and is reviewed in (Bouillon et al., 2008). $1,25(OH)_2D$ induces the expression of the enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24), which catabolizes both 25(OH)D and $1,25(OH)_2D$ into biologically inactive, water-soluble calcitroic acid (Holick, 2007), (Prosser and Jones, 2004). Mice with targeted gene mutations in CYP24 have an excess of endogenous $1,25(OH)_2D$ and perinatal lethality in half of newborns (St-Arnaud et al., 2000). This metabolite circulates at approximately 1000-fold lower concentrations than 25(OH)D and is generally present at 20 to 65 pg/ml in normal human plasma (Cannell and Hollis, 2008).

25(OH)D is uniquely metabolized in the kidney (Fraser and Kodicek, 1970), but the same enzyme is expressed in a variety of other tissues such as bone, placenta, macrophages, skin, brain, pancreas, as well as in prostate, breast, colon, lung, pancreatic β cells, monocytes, and parathyroid cells. 25(OH)D regulates CYP27B1 functions of locally produced $1,25(OH)_2D$ (Bikle, 2007; Buell and Dawson-Hughes, 2008; Garcion et al., 2002; Hewison et al., 2004; Jones, 2007; Kiraly et al., 2006; Lechner, Kallay, and Cross, 2007; Lou et al., 2004; van Driel et al., 2006; Vigano et al., 2006; Zehnder et al., 2001) to form $1,25(OH)_2D$ for autocrine and paracrine action. Local CYPB1 regulation is different compared to immunogenic stimuli, and not calcium regulating, as reviewed in (Bouillon et al., 2008). Homozygous mutations in the CYPB1 gene causes disease, which is described as VD resistant rickets type I (Prader, Illig, and Heierli, 1961). These mice and humans have severe rickets, which is curable with a high intake of 25(OH)D or normal 1α-hydroxylated VD replacement therapy (St-Arnaud et al., 1997; Takeyama et al., 1997).

Transport of lipophilic VD metabolites to the blood occurs through VD binding proteins (DBP). DBP is a serum protein and like albumin, is expressed and secreted by the liver. Plasma concentration of DBP is 20 times higher than the total amount of VD metabolites, of which 99 % of them are protein bound. It is generally believed that free fractions of $1,25(OH)_2D$ enter target cells by diffusion and then, interact with cytoplasmic/nuclear VD receptors (Haussler et al., 1998; Sutton and MacDonald, 2003). Protein bound VD

metabolites have limited access to target cells and hence an increased half-life circulation. Therefore DBP protects the tissues from toxic levels of VD. However, free $1,25(OH)_2D$ concentration remains constant even if DBP levels change as a result of the tight self-regulation of VD metabolism. However, DVD-KO mouse had a reduction of $1,25(OH)_2D$ in total levels, but tissue levels were unaffected (Zella et al., 2008). DBP deficient mice are prone to developing a VD deficiency when fed a VD deficient diet (Cooke and Haddad, 1989). 25(OH)D entry into renal proximal convoluted tubular cells occurs through receptor-mediated uptake, called endocytosis-a megalin facilitated process (Megalin is part of a set of complex proteins that facilitate endocytosis) (Nykjaer et al., 1999).

Megalin deficient mice cannot reabsorb neither DBP nor 25(OH)D into nephrons, and therefore develop VD-deficiency and rickets (Nykjaer et al., 1999). Inside renal tubular cells, DBP is degraded and 25(OH)D is released for metabolism by 1α or 24 hydroxylases. There are many intracellular D binding proteins (IDPB), which bind VD and regulate their intracellular metabolism (Tanaka and Deluca, 1973). IDBPs are homologues of human heat shock proteins (HSP) (Gacad and Adams, 1998). IDBP-1 and IDBP-3 both interact with megalin (Adams et al., 2003), increase the movement of 25(OH)D into mitochondria for hydroxylation, and further transport of calcitriol to the VDR (Wu et al., 2002). Megalin levels are increased by $1,25(OH)_2D$ (Liu et al., 1998).

VD is required throughout life to keep calcium and phosphorus homeostasis in the body and many other important biological functions (Bikle, 2007; Bouillon et al., 2008; Cannell and Hollis, 2008; Holick, 2007). Geography, time of year, pollution, cloud cover and use of sunscreens and consumption of VD poor products all cause low plasma level of 25(OH)D to below 10-15 ng/mL. Only a few people today achieve the normal level, which is defined as 30 to 40 ng/mL (Cannell and Hollis, 2008; Holick, 2007; Ravin, 2008).

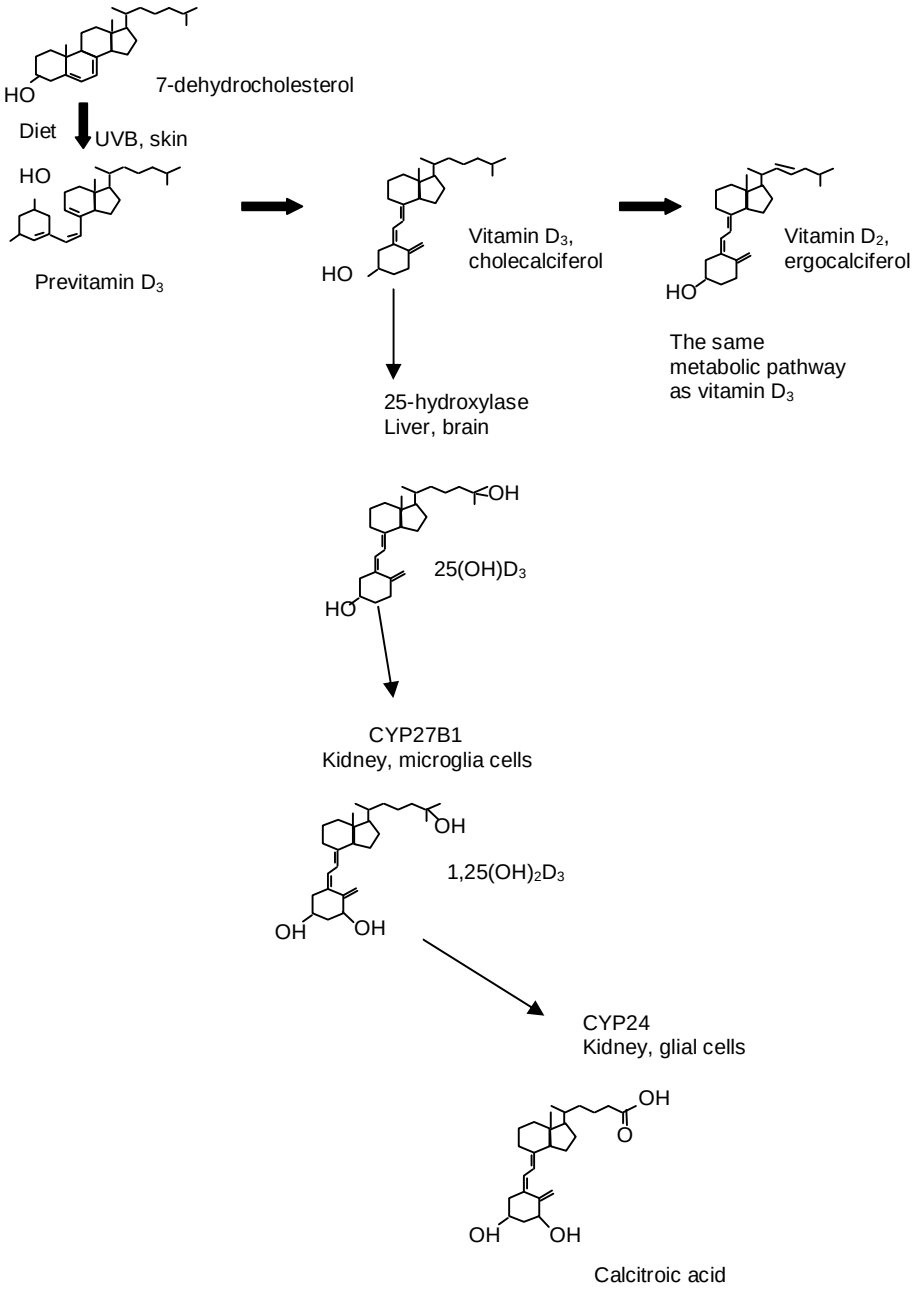

Figure 1. Metabolism of vitamin D. Vitamin D is derived from diet or from ultraviolet (UVB) light and undergoes induced conversion through skin cellular plasma membrane 7 –dehydrocholesterol into provitamin D followed by thermal isomerization into vitamin D_3 (cholecalciferol). First, it is 25-hydroxylated in the liver and 1 alpha-hydroxylated in the kidney to give $1,25(OH)_2\ D_3$. The two enzymes involved in the bioactivation process are 25-hydroxylase and 1 alpha-hydroxylase. The presence in brain cells of the enzymes involved in this activation process suggest that $1,25(OH)_2\ D_3$ metabolism can also occur in the brain. Inactivation of $1,25(OH)_2\ D_3$ occurs through a series of oxidation reactions at carbon 24 and 23, leading to side chain cleavage and the formation of biologically inert calcitroic acid.

5.1.3 Vitamin D receptors

Most biological activities of VD metabolites occur through nuclear protein VD receptor (VDR), which is ligand-inducible transcription factor belonging to nuclear receptor superfamily of steroid and thyroid hormones (Baker et al., 1988; Brown, Dusso, and Slatopolsky, 1999; Carlberg and Seuter, 2007; McDonnell et al., 1987). Calcidiol and $24,25(OH)_2D$ bind to VDR almost 100 times less than calcitriol (Mellon and DeLuca, 1979). The mammalian forms of the VDR protein range in molecular weight between 52-60 kD by biochemical analysis, although the calculated molecular weight deduced from the amino acid sequence is 48,3 kD (Issa, Leong, and Eisman, 1998). VDR can be divided by function into several domains, typical for all steroid receptors (Fig. 2A) (Haussler et al., 1998; Jones, Strugnell, and DeLuca, 1998). The A/B domain is located at the N-terminus, is short and hyper-variable among nuclear receptors, and helps determine the transactivation capacity of VDR (Jurutka et al., 2000). The C domain or DNA binding domain is similar among nuclear receptors and contains two zinc fingers interacting with a DNA specific sequence of the target gene (McDonnell et al., 1989). The D domain is a highly flexible hinge region, which due to its rotational flexibility allows receptor dimerization and interaction with DNA (Mangelsdorf et al., 1995). The E or E/F domain located at the COOH-terminal portion of the VDR molecule contains the ligand binding domain (LBD), which is a multifunctional globular domain that mediates selective interactions of the receptor with hormones (McDonnell et al., 1989), and RXR nuclear receptor partners (Nakajima et al., 1994), as well as with co-modulatory proteins (Masuyama et al., 1997). The structure of the VDR LBD is similar to retinoic acid receptor (RXR) (Rochel et al., 2000). LBD is organized into 13 α-helices (H1, H2, H2*n*-H12) and 3 ß-sheets, forming a hydrophobic ligand-binding pocket. Ligand binding to VDR causes a conformational change of active function-2 (AF-2) fragment (helix 12 is in this region). This is crucial for the activation of transcription through recruiting transcription factors by co-regulators. Mutation of AF-2 cause the inactivity of nuclear receptor transcription, despite retaining the ability to bind ligand (Danielian et al., 1992; Masuyama et al., 1997; Nakajima et al., 1994).

Naturally occurring mutations in the human VDR are found in patients with hereditary VD-resistant rickets (HVDRR). Patients with this disorder have reduced or no response to VD. The VDR KO mouse provides an animal model of HVDRR (Yoshizawa et al., 1997). Most of the VDR mutations maintain DNA binding ability, however transactivation is affected (Malloy et al., 1997; Malloy, Weisman, and Feldman, 1994; Saijo et al., 1991). The second most common mutation results in a truncated LBD that lacks ligand-binding activity (Kristjansson et al., 1993; Wiese et al., 1993). The third class comprises mutations in the extreme C-terminus and affects receptor heterodimerization and therefore calcitriol inducible gene transactivation (Whitfield et al., 1996).

Human VDR is a product of a single chromosomal gene, located at 12q13-14 (Faraco et al., 1989; Szpirer et al., 1991). Structurally, mouse VDR has homology between chickens, humans and rats. The C fragment has 100 % homology with the rat and human VDR gene, as does the E domain (Kamei et al., 1995).

A.

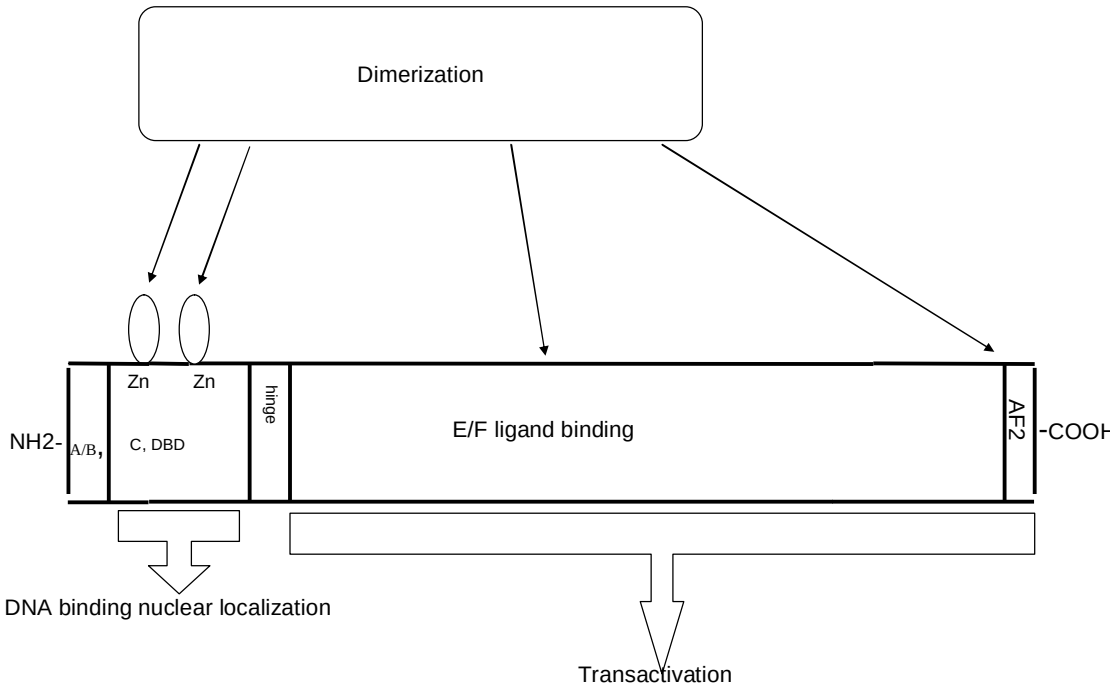

Figure 2A. Structural domains of VDR. Functional domain structure of the human VDR molecule. The N terminal region is short relative to other steroid hormone receptors. This region is followed by two zinc fingers, which constitute the principal DNA binding domain. Nuclear localization signals (NLS) are found within and close to the C-terminal of the DNA binding domain. The ligand binding domain makes up the bulk of the C-terminal part of the molecule, with the AF2 domain comprising the most C-terminal region. The AF2 domain is largely responsible for binding to co-activators such as the SRC family and DRIP. Regions on the second zinc finger and within the ligand binding domain facilitate heterodimerization with RXR. Corepressor binding is less well characterized, but appears to occur in the ligand binding domain.

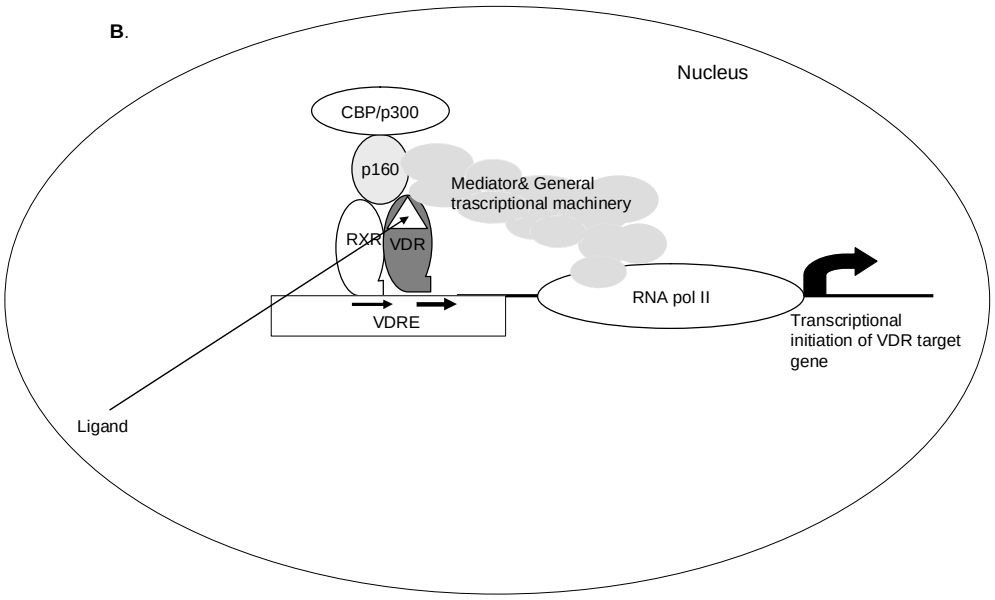

Figure 2B. Regulation of VDR transactivation. Ligand-initiated gene transcription. Ligand, 1,25$(OH)_2$D enters the target cell and binds to its receptor, VDR. The VDR then heterodimerizes with the retinoid X receptor (RXR). This increases the affinity of the VDR/RXR complex for the vitamin D response element (VDRE), a specific sequence of nucleotides in the promoter region of the vitamin D responsive gene. Binding of the VDR/RXR complex to the VDRE attracts a complex of proteins termed nuclear receptor coactivators (p160), and transcriptional coactivator (CBP/p300) or mediator complex to facilitate changes in chromatin architecture and to enhance RNA polymerase entry. Transcription of the gene is initiated to produce the corresponding mRNA, which leaves the nucleus to be translated into the corresponding protein (Modified from (Rosenfeld, Lunyak, and Glass, 2006)).

Binding of 1,25$(OH)_2$D to VDR modulates macromolecular interactions, leading to transcription of selected target genes (Haussler et al., 1998) (summarized in Fig. 2B). In response to binding, ligands associate with VDR and promotes its heterodimerization with retinoid X receptor (RXR), a common heterodimmeric partner for other nuclear trans retinoic receptors (Mangelsdorf and Evans, 1995). Modulation of gene expression is not directly mediated by binding VDR/RXR heterodimers to DNA, but rather is dependent upon the ability of this dimer to recruit co-regulatory protein complexes, not silencing by co-repressors (Glass and Rosenfeld, 2000; McKenna, Lanz, and O'Malley, 1999; McKenna and O'Malley, 2002).

Unliganded VDR is likely kept transcriptionally silent, even when present it in the nucleus and bound to chromatin by one or more co-repressors. Corepressors like silent mediators for retinoid and thyroid hormone receptors (SMRT), nuclear receptor corepressors (Ncor) and Alien are known to regulate VDR action (Polly et al., 2000; Tagami et al., 1998). Co-regulators contribute to histone modification, chromatin remodeling, recruitment of RNA polymerase (which recruits transcription factors and induces the target genes (Rosenfeld, Lunyak, and Glass, 2006; Spencer et al., 1997)), and ATP-dependent chromatin remodeling (Li, Carey, and Workman, 2007; Villagra et al., 2006). VDR not only directly

activate gene transcription, but also directly down-regulate the transcription of genes of the parathyroid hormone, CYP27B1 (Kim et al., 2007; Murayama et al., 1998). The heterodimer VDR-RXR or VDR homodimers are bound to VD response elements (VDRE) in the promoters of target genes, where VDR occupies the 5`-located half-site (Demay et al., 1992) or through E-box-type elements in human PTH promoters (Kim et al., 2007) and in 1 α-OH (Murayama et al., 2004). VDREs are composed of two hexameric repeats with an intervening spacer of three nucleotides (DR-3 elements). Therefore, the target gene of $1,25(OH)_2D$ is conferred on one hand through ligand binding VDR-RXR heterodimerization, and also high-affinity binding to DR-3 VDREs. Other VDRE type is DR4 is a direct repeat intercepted by 4 nucleotides. (Yen et al., 1996)

Studies in mice with deletions in the VD /VDR system revealed VDR may have ligand-independent functions, especially in the skin and immune system (Bouillon et al., 2008). Several studies indicate that VD metabolites may exert rapid effects (seconds to minutes). In chick intestine, 24,25-dihydroxyvitamin D rapidly suppresses the actions of $1,25(OH)_2D$ and PTH on calcium transport (Nemere, 1999). Since the rapid effect occurred in osteoblasts (Baran, 1994; Baran et al., 1992), it occurred through a non-genomic signal transduction pathway, such as the activity of protein kinase C (PKC) and regulation of ion channels (Fleet, 2004; Nemere et al., 1998). The second class of receptors for VD metabolites has been described as being present in the plasma membrane of several cell types (Nemere et al., 1994). This membrane receptor for $1,25(OH)_2D$ has been designated as membrane VDR receptor (VDR mem). VDR mem was found in chick brain (Jia and Nemere, 1999) and its molecular weight was estimated to be 60 kDa (Nemere et al., 1994), however this receptor must be designated as “putative,” since it has not yet been cloned to reveal its biochemical structure (Losel and Wehling, 2003; Norman, 1998; Norman, 2006; Norman et al., 2002). Studies continuously occur in this field, and VDR mem was also found in heart cells (Tishkoff et al., 2008). Moreover, nuclear VDR was discovered to have a role in non-genomic actions (Nemere et al., 1994; Nemere et al., 1998; Norman, 2006), although the exact mechanisms remain unknown.

Human VDR genes contain many allelic variants, including Cdx-2, FokI, BsmI, ApaI and TaqI, the most commonly investigated and associated with a number of phenotypes, such as bone mineral density, fracture risk and cancer (Uitterlinden et al., 2004). In addition, genetic variance in the VDR gene influences susceptibility to age-related changes in cognition and depressive functions (Kuningas et al., 2007).

5.1.4 The vitamin D endocrine system and physiological functions

The VD endocrine system characterizes the classical functions of VD in the responsive organs, such as kidney, bone, parathyroid gland, and intestine where it regulates calcium homeostasis and bone development. VDR is expressed during embryonic development in tissues that are involved in calcium and phosphate regulation, as well as bone development (Dusso, Brown, and Slatopolsky, 2005).

Although VDR KO mice are phenotypically normal at birth, they develop severe rickets and an osteomalasia phenotype after weaning (Erben et al., 2002; Li et al., 1997; Yoshizawa et al., 1997). Mice deficient in 1 α-OH displayed a similar phenotype, with respect to bone phenotype and low calcium plasma levels (Dardenne et al., 2001; Panda et al., 2001b). VD increases the absorption of dietary calcium and phosphate in intestine and reabsorption in the kidneys. The transcellular calcium transport involves three steps: (i) calcium influx into

epithelial cells, mediated by apical calcium channels (epithelial calcium channels: transient receptor potential vanilloid (TRPV) family; TRPV 5 EcaC1, and TRPV 6 or CaT1, EcaC2), which is promoted by an electrochemical gradient; (ii) Thereafter, calcium binding proteins (calbindins (CaBPs), namely, calbindin D9k) perform intracellular calcium transferring; (iii) Finally, the basolateral plasma membrane ATPase (PMCA1) pumps calcium from the cell into extracellular fluid using an energy-requiring process (Bouillon, Van Cromphaut, and Carmeliet, 2003).

In the kidney, Ca^{++} reabsorption takes place in the distal nephron. In kidney TRPV5 is the main apical calcium channel, but TRPV6 is the main channel in duodenum. In the intestine the only cytosolic calcium transporter is CABP-9k, but in kidney both CABP-9k and CABP-28k are present; the $PMCA_{1b}$ in intestine and Na^{+}/Ca^{++} exchanger (NCX1) in kidney regulate calcium extrusion (Hoenderop et al., 2000; Hoenderop, Nilius, and Bindels, 2005). In adult VDR KO mice intestinal calcium absorption was reduced (Song, Kato, and Fleet, 2003; Van Cromphaut et al., 2001). The decrease in intestinal calcium absorption is associated with altered expression of calcium transport proteins in duodenum of VDR or 1 α-OH KO mice. TRPV 6 mRNA levels were low in VDR KO mice (Van Cromphaut et al., 2001). CABP-9k mRNA and protein levels were reduced in different VDR KO strains (Bolt et al., 2005; Li, Pirro, and Demay, 1998; Van Cromphaut et al., 2003). Also, aging reduces Ca^{++} absorption, as TRPV 6 (van Abel et al., 2006) and CABP-9k mRNA and protein levels decline with age in WT mice but not in VDR KO mice (Li et al., 2001). A rescue diet (consisting of high lactose and high calcium) increases the expression of the calcium binding protein calbindin D9k in normal mice, but not in VDR KO mice (Bolt et al., 2005) and mRNA expression of renal and duodenal calbindin-D9k is reduced in both the lungs and brain (Li et al., 1998), indicating that functional VDR is required to moderate dietary suppression of calbindin. 1α-OH KO mice also showed a decrease in CaBP-9k expression (Dardenne et al., 2001; Panda et al., 2001b).

Other studies showed that a high 2% Ca^{++} rescue diet in VDR and 1α-OH KO mice can normalize blood Ca^{++} levels (Hoenderop et al., 2002; Song, Kato, and Fleet, 2003). Although ablation of TRPV6 and CABP-9k did not severely change intestinal calcium absorption, when calcium intake was sufficient or when TPRV6 was deleted, there was an effect when a low calcium diet was used (Benn et al., 2008). These data indicate that VDR promotes sufficient intestinal calcium absorption, by regulating several calcium transport proteins, including proteins which form paracellular calcium channels (Fujita et al., 2008).

VD/VDR actions are involved in the active reabsorption of calcium in the distal tubule. VDR KO mice showed high urinary calcium excretion, suggesting disturbed calcium reabsorption. VDR KO mice raised on a rescue diet exhibited a significant increase in the calcium/creatinine ratio compared to WT mice (Li et al., 2001). Normalization of calcitriol serum levels in 1α-OH KO mice resulted in increased expression of renal calcium proteins and serum calcium levels (Hoenderop et al., 2002). Calcitriol regulates Ca^{++} renal reabsorption via regulation of EcaC expression in the kidneys (Hoenderop et al., 2001; Hoenderop, Willems, and Bindels, 2000). VDR action is required for adequate calcium reabsorption in the kidney, by regulating the expression of several calcium transports, where TRPV5 plays a crucial role (Renkema et al., 2005; Zheng et al., 2004).

VDR KO and 1α-OH KO mice are also characterized by parathyroid hyperplasia increased PTH mRNA in parathyroids and elevated serum PTH levels. Feeding these mice a rescue diet normalized serum PTH levels, indicating that Ca^{++} plays a crucial role in the regulation

of phosphate (Li et al., 1998; Van Cromphaut et al., 2001). Regulation of PTH production is negatively regulated by serum ionized calcium levels through the calcium-sensing receptor in the parathyroid gland, which stimulates PTH secretion in low Ca^{++} conditions. This increases Ca^{++} reabsorption from kidney and bone through the activation of 1α-OH (Dusso, Brown, and Slatopolsky, 2005). Parathyroid gland hyperplasia was delayed in 1α-OH KO mice when they were treated with $1,25(OH)_2D$ (Panda et al., 2004). The double PTH x 1α-OH KO mice died at 3 weeks with tetany, due to severe hypocalcaemia, suggesting that secondary hyperparathyroidism contributes in controlling calcium homeostasis (Xue et al., 2005).

$1,25(OH)_2D$ regulates Na-Pi protein expression in the intestine and kidney (Fig 3) (Carling et al., 1995; Hattenhauer et al., 1999). These apical cotransporters, Na/Pi-2a and Na/Pi-2b, mediate phosphate uptake in the proximal renal tubules and small intestine (Hattenhauer et al., 1999). Another factor which regulates phosphate homeostasis is circulating FGF23, which is produced by osteoblastic cells. The 1, $25(OH)_2D$/VDR pathway induces the expression of FGF23 (Kolek et al., 2005) and VDR KO mice showed an undetectable level of FGF23 (Shimada et al., 2005). FGF23 levels increased with a rescue diet in VDR KO mice (Shimada et al., 2005), suggesting that FGF23 expression is also regulated by a VDR-independent pathway. Treatment of VDR KO mice with FGF23 decreased hypophosphatemia (Inoue et al., 2005; Miyamoto et al., 2005). FGF23 levels are regulated by klotho protein, klotho KO mice had severely increased FGF23 serum levels, although they showed hyperphosphatemia and hypervitaminosis D due to increased expression of Na/Pi-2a and 1 α-OH (Razzaque and Lanske, 2006; Razzaque et al., 2006). Klotho deficient mice fed a VD deficient diet showed a significant rescue of this phenotype (Tsujikawa et al., 2003).

VD is important for normal development and the mineralization of the skeleton, since VD deficiency causes rickets in young and osteomalasia in adult animals. The VDR and 1α-OH KO mice show a rickets phenotype (Dardenne et al., 2001; Kato et al., 1999; Panda et al., 2001a; Yoshizawa et al., 1997), which could be rescued with a high-calcium, high-P, and high-lactose diet (rescue diet) (Dardenne et al., 2004; Li et al., 1998), as seen in human VD-dependent rickets type II and I. Thus the abnormalities were less severe by normalizing Ca^{++} plasma level. The rescue diet did not completely normalize the growth plate, because the $1,25\ (OH)_2D$–VDR system is essential for the normal coupling of bone remodelling (Panda et al., 2004). After weaning, VDR KO mice had an expansion of the growth plate due to impaired apoptosis of hypertrophic chondrocytes (Donohue and Demay, 2002). Normalization of the phenotype in mice with VDR or 1 α-OH deficiency through a high calcium and lactose diet are strong arguments for intestinal epithelium being the primary target for VD action (Bouillon et al., 2008).

Numerous data indicate that many tissues have VDR and respond to $1,25(OH)_2D$ in ways that are not directly related to Ca^{++} homeostasis. Many of these tissues are able to convert circulating 25(OH)D into $1,25(OH)_2D$ (Bouillon et al., 2008). The protective role of VD has been shown in prostate, breast, and colon cancer through the control of cell differentiation and proliferation (Tuohimaa et al., 2005; Wactawski-Wende et al., 2006; Zinser, Suckow, and Welsh, 2005). The skin is the most obvious target for the VD/VDR endocrine system, as was shown in all VDR KO mice strains and patients with hereditary VD-resistant rickets, or vitamin-deficient rickets type I where serum calcium normalization fails to correct alopecia, dilated hair follicules and dermal cysts (Li, Pirro, and Demay, 1998). Moreover,

VDR is a potent repressor of the hairless (Hr) gene (Hsieh et al., 2003) and antiproliferative properties in psoriatic keratinocytes (Cordero et al., 2002).

$1,25(OH)_2D$ and VDR control heart functions and blood pressure (Li et al., 2002; Simpson, Hershey, and Nibbelink, 2007). VDR and 1 α-OH KO mice overexpress renin, which causes systemic hypertension and cardiac hypertrophy (Li et al., 2002). $1,25(OH)_2D$ and VDR regulate skeletal muscle growth and differentiation (Boland, 1986; Endo et al., 2003). Importantly, VD is a modulator of immune responses such as controlling infections and autoimmune diseases (Bouillon et al., 2008; Dusso, Brown, and Slatopolsky, 2005). Finally, VDR is widely expressed in the brain and the peripheral nervous system (Eyles et al., 2003; Eyles et al., 2005; Garcion et al., 2002; Kiraly et al., 2006), indicating the important role of VD/VDR in the regulation of the nervous system functions.

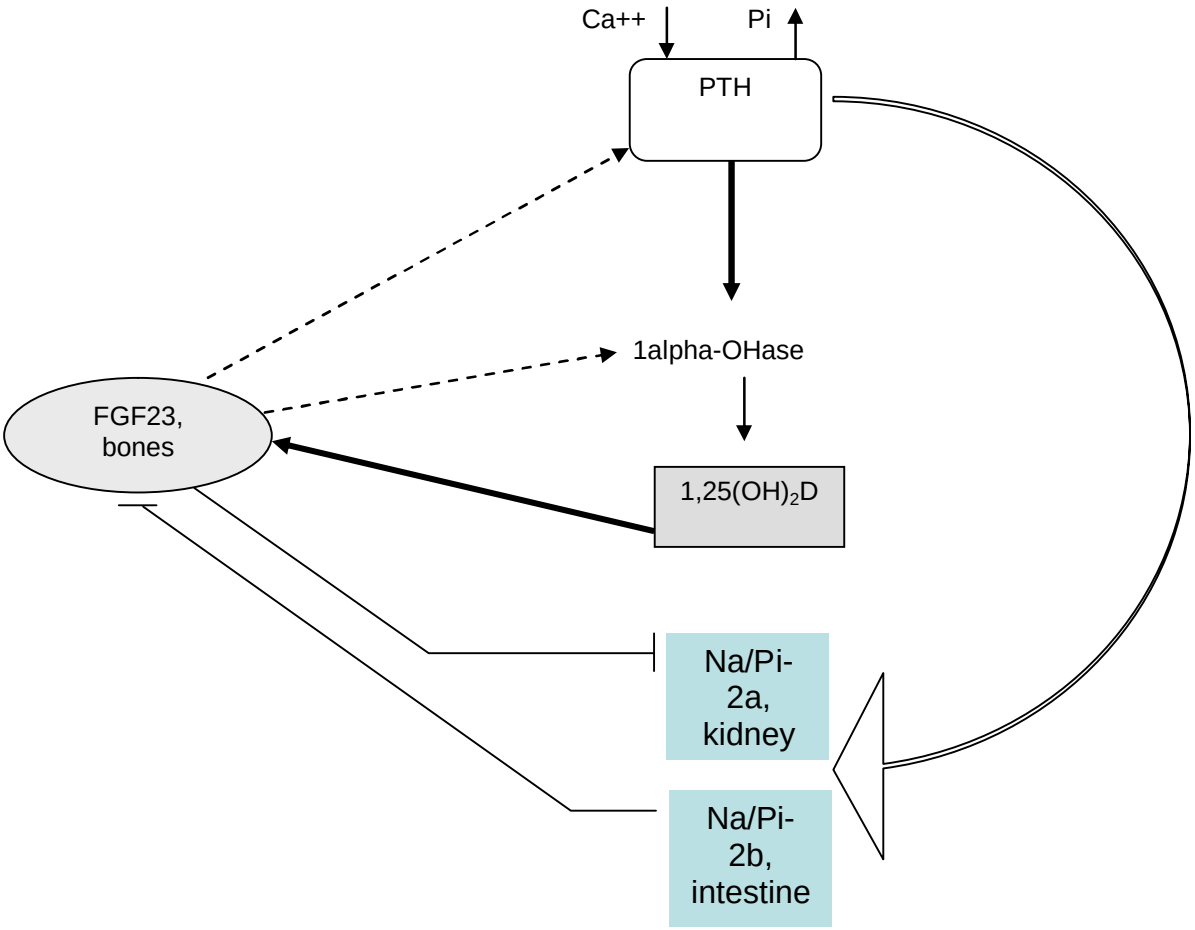

Figure 3. Effects of the parathyroid gland and FGF23 on phosphate homeostasis. Hypocalcaemia and hypoposphatemiainduce induce PTH secretion, stimulating renal calcitriol synthesis. Increased calcitriol levels upregulate FGF23. Increased PTH and FGF23 levels reduce Na/Pi expression in kidney and intestine, thus leading to reduced renal reabsorption and therefore lower Pi levels. FGF23 downregulates renal 1alpha-hydroxylase and decreases PTH secretion. Modified from Bouillon et al 2008.

5.2 EFFECTS OF VITAMIN D ON THE NERVOUS SYSTEM

The role of VD in the nervous system is currently getting more attention. VDR is expressed in most brain areas. Patients suffering from rickets show no obvious nervous system dysfunction, but VDR KO mice and VD deficient rodents display a variety of behavioral abnormalities (discussed in sections 5.4, and 5.6.1). The brain localization of 25(OH) and 1α-OH enzymes and distribution of VDR suggest that VD may have autocrine/paracrine properties by locally performing bioactivation of the VD prohormone (Eyles et al., 2005; Garcion et al., 2002; Stumpf et al., 1982; Zehnder et al., 2001). $1,25(OH)_2D$ has been detected in the cerebrospinal fluid, and this hormone has been shown to cross the blood-brain barrier (Balabanova et al., 1984; Gascon-Barre and Huet, 1983; Pardridge, Sakiyama, and Coty, 1985), although its uptake is limited (Gascon-Barre and Huet, 1983; Pardridge, Sakiyama, and Coty, 1985).The sections below discover the role of VD target genes in the brain from several cell studies of rodents and humans. The involvement of VD in neuronal differentiation and brain function is also discussed. Rodent data indicates a role of VD in the fetal and adult brains. Human data of VD effects on early brain development has not been fully explored.

5.2.1 Distribution of VDR and 1,α-hydroxylase throughout the CNS. Specific functions of VD in the brain

Evidence for a role of VD in brain function began to accumulate over two decades ago, with autoradiographic studies of rodent brain with ^{3}H-labeled $1,25(OH)_2D$ demonstrating target sites for VD in certain neurons of forebrain, hindbrain, spinal cord and sensory ganglion (Stumpf et al., 1988; Stumpf et al., 1982). Both autoradiographic studies and immunohistochemistry showed the presence of VDR in pituitary cells (Stumpf, Sar, and O'Brien, 1987) and mRNA and protein VDR expression in human pituitary gland (Perez-Fernandez et al., 1997), suggesting a possible role of VD in regulation of the brain endocrine system. *In vitro* and animal data confirm the presence of VDR and 1α-OH in the brain (Eyles et al., 2005; Zehnder et al., 2001) and further animal studies revealed the presence of VDR in the mature and developmental brain (Brown et al., 2003; Johnson et al., 1996; Langub et al., 2001; Luine, Sonnenberg, and Christakos, 1987; Musiol et al., 1992; Taniura et al., 2006; Walbert, Jirikowski, and Prufer, 2001; Veenstra et al., 1998). Immunohistochemical studies revealed that the distribution of VDR in human and rodent brains is rather similar (Eyles et al., 2005). VDR have been found in microglia, astrocytes, oligodentrocytes and Schwann cells (Baas et al., 2000; Cornet et al., 1998; Langub et al., 2001; Naveilhan et al., 1993; Neveu et al., 1994; Prufer et al., 1999). These data indicate a role of VD in the peripheral nervous system (Johnson et al., 1996). The presence of VDR in the limbic system, cortex, cerebellum of rodents and humans (Eyles et al., 2005; Langub et al., 2001; Musiol et al., 1992; Walbert, Jirikowski, and Prufer, 2001) support a functional role for VD in the regulation of behavior and cognitive functions. It is also consistent with the distribution of other neurosteroids (Prufer and Jirikowski, 1997). VDR is found in the olfactory, visual and auditory sensory systems (Glaser et al., 1999; Prufer et al., 1999; Zou et al., 2008), suggesting that the somatosensory system is also a target of $1,25(OH)_2D$.

VDR like immunoreactivity was found in the nucleus vestibularis, which extends its efferents to cerebellar Purkinje cells and the thalamic part of the vestibular system, nucleus ventrolateralis, suggesting that the vestibular system is also a target of VD (Prufer et al., 1999). Expression of VDR in motor neurons (Prufer et al., 1999) suggests its role in

regulation of motor functions. A putative receptor for 1,25$(OH)_2$D has been detected in chick brain (Jia and Nemere, 1999), allowing speculation that 1,25$(OH)_2$D could act like other neuroactive hormones in modulating neuronal activity and neurotransmitter receptors (Rupprecht and Holsboer, 1999; Zakon, 1998). It is of particular importance that VDR and catalytic enzymes are colocalized in the brain (Baulieu, 1998; Melcangi and Panzica, 2001), supporting an autocrine/paracrine function for VD. These findings support a functional role for VD in the human brain (McGrath et al., 2001).

5.2.2 Neuroprotective effects of 1,25$(OH)_2$D

Studies have shown that VD confers regulatory benefits in neuronal Ca^{++} homeostasis and protects neurons from excess calcium entry in the brain (Brewer et al., 2001). Regulation of brain calcium homeostasis occurs via down-regulation of the L-type voltage-sensitive Ca^{++} channels (L-VSCCs) in hippocampal cultured neurons, thus contributing to protection from excitotoxic cell death (Brewer et al., 2001) . Treatment with 1,25$(OH)_2$D in aged rats restores aging neurons (Brewer et al., 2006). These beneficial changes protect neurons during ischemic events or excitotoxic insults. Neuroprotective effect of 1,25$(OH)_2$D may also happen through reduction of Ca^{++} toxicity by stimulation of expression of Ca^{-}binding proteins (de Viragh, Haglid, and Celio, 1989), thus supporting the idea that VD regulates neuronal homeostasis and may prevent neuronal aging.

Aging is the most common risk factor for the development of neurodegenerative disease. Hippocampal cell loss and neuronal aging have been attributed to elevated L-type voltage calcium channel density and glucocorticoid (GC) neurotoxicity (Kimura et al., 1998). 1,25$(OH)_2$D participates in modulation of CNS detoxification pathways through regulation of γ-Glutamyl transpeptidase (γ-GT), an enzyme which up-regulates the glutathione (GTH) pool and reduces the production of reactive nitrogen species in astrocytes (Garcion et al., 1999; Garcion et al., 1996). Increased brain GTH levels through 1,25$(OH)_2$D also protects against toxicity in cultured mesencephalic neurons (Ibi et al., 2001; Shinpo et al., 2000). γ-GT is involved in the elimination of reactive oxygen species (ROS). Oxidative stress may also contribute to the pathophysiology of neurodegenerative disorders (Buell and Dawson-Hughes, 2008; McGrath et al., 2001). This data suggest that VD could be useful in the treatment of neurodegenerative disease (Cass, Smith, and Peters, 2006).

1,25$(OH)_2$D has also been shown to be a potent immunomodulator (Garcion et al., 2002; Kiraly et al., 2006) . VD may inhibit the synthesis of inducible nitric oxide synthase (iNOS) (Garcion et al., 1997; Garcion et al., 1998), an enzyme which is produced in response to inflammatory stimuli (Shinpo et al., 2000) and have harmful effects on the CNS (Dawson and Dawson, 1996; Merrill et al., 1993). 1,25$(OH)_2$D has immunosuppressive effects in various models of autoimmune disease (Cantorna, Hayes, and DeLuca, 1996; Cantorna et al., 1998; Nataf et al., 1996). VD may protect the structure and integrity of neurons through neurotrophin (NT) synthesis and nerve growth factor (NGF) (Cornet et al., 1998; Naveilhan et al., 1996; Neveu et al., 1994), and regulate trophic factors such as glial cell derived neurotrophic factor (GDNF) in cortex and striatum (Wang et al., 2000), as well as brain cancer cells (Naveilhan et al., 1996). NT is a protein necessary for neuronal survival in aging and neuropathological conditions (Woo and Lu, 2006). In animal models, treatment with calcitriol increased GDNF and reduced oxidative stress in Parkinson's disease (Wang et al., 2001). VD depletion in utero resulted in reduced levels of NGF and GDNF and morphological brain changes in newborn rodents, which remained throughout adulthood

(Becker et al., 2005). Furthermore, the anticancer activity of 1,25$(OH)_2$D in the brain has also been reported (Baudet et al., 1996; Davoust et al., 1998; Garcion et al., 2002; Kiraly et al., 2006; Magrassi et al., 1992; Naveilhan et al., 1994; Naveilhan et al., 1996).

5.3 EXPERIMENTAL SEIZURES

Chemically induced seizures are widely used as a model for epileptogenesis, including experimental epilepsy evoked by pentylenetetrazole, pilocarpine or kainate (Barton and Shannon, 2005; Pitkanen, 2002; Soderpalm, 2002).

5.3.1 Anticonvulsant action of vitamin D

It is well known that VD deficiency is associated with epilepsy, and 25(OH)D levels are low in epilepsy patients (Camadoo, Tibbott, and Isaza, 2007; Erdeve et al., 2007; Mintzer et al., 2006; Nicolaidou et al., 2006; Offermann, Pinto, and Kruse, 1979; Williams et al., 1984). Antiepileptic drugs are associated with low bone density (El-Hajj Fuleihan et al., 2008; Vestergaard, 2008) and severe VD deficiency in patients with rickets, and osteomalacia is associated with hypocalcaemic seizures (Orbak et al., 2007). Hypocalcaemia and hyperparathyroidism may cause convulsions, leading to a loss of seizure control (Ali et al., 2004; Armelisasso et al., 2004; Bindu and Harinarayana, 2006; Gupta and Grover, 1977). Other observations in patients with hypoparathyroidism show gradual cognitive impairment and generalized tonic clonic epilepsy (Titlic et al., 2008). The direct anticonvulsant properties of calcitriol have been shown by Siegel et al 1984 (Siegel et al., 1984), as calcitriol significantly increases the hippocampal electroconvulsive threshold in rats. At the molecular level, the expression of VDR mRNA in the hippocampal formation of rats was increased in pilocarpine-induced seizures (Janjoppi et al., 2008). A recent study demonstrated that calcitriol may enhance the anticonvulsant effects of several antiepileptic drugs (Borowicz et al., 2007). The low-calcemic analog of calcitriol, PRI-2191, was able to prevent seizure-related changes in the peripheral immune system and brain (Tetich et al., 2005). This suggests that the VD/VDR endocrine system has direct or indirect effects on epileptic activity and may play an important role in the regulation of epileptogenesis.

5.4 ANIMAL EXPERIMENTS

Numerous studies have been performed with mice and rats to examine the effects of maternal low VD on offspring brain development (McGrath et al., 2004b), expression of genes and the effects of a maternal VD restricted diet on the behavioral performance of adult animals (McCann and Ames, 2008).

VD deficiency during early life has neurobehavioral effects, as the brains of newborn rats born to VD deficient (DVD) mothers are larger, have a longer cortex and lateral ventricle volume, but a thinner neocortex and increased brain cell proliferation. The expression of NGF and GDNF was decreased in DVD newborn rats (Eyles et al., 2003). These effects were not transient, at 10 weeks of age the lateral ventricles remained large and the expression of genes involved in neuronal function (NGF, GABA-A) were lower in the brain of transiently VD deficient rats during early development (Eyles et al., 2007; Feron et al., 2005). *In vitro* studies suggest VD is a potent inhibitor of neuronal mitosis and increases neuron outgrowth and the amount of NGF in embryonic brain (Brown et al., 2003).

Analysis of prefrontal and hippocampal areas of brain from rats exposed to transient prenatal DVD, identified dysregulation of proteins involved in a variety of cellular functions, half of them expressed in schizophrenia or multiple sclerosis (MS) (Almeras et al., 2007). VD deficiency in rodents has significant effects also in late life. In rats and mice these suggest behavioral abnormalities like hyperlocomotion in the hole board test and open field tests, and enhanced exploration in elevated plus maze (Burne et al., 2004a; Burne et al., 2004b; Harms et al., 2008). DVD-deficient rats showed persistent mild learning and memory dysfunction (Becker et al., 2005; Burne et al., 2004a; Burne et al., 2004b), and hyperlocomotion (Kesby et al., 2006). Alterations in behavioral performance did not occur due to alterations in stress responses or through the hypothalamic pituitary axis (HPA) (Eyles et al., 2006). These suggest that prenatal VD deficiency may alter adult brain and be associated with schizophrenia (Kesby et al., 2006; McGrath et al., 2008; McGrath et al., 2004a) .

The neuroprotective effects of calcitriol have also been demonstrated in rodent models of stroke and 6-hydroxy-dopamine toxicity (Wang et al., 2001; Wang et al., 2000). In rats calcitriol increased convulsive threshold activity (Siegel et al., 1984). Rodent data indicates that perinatal DVD can result in long lasting changes in gene and protein expression, and may be associated with structural and behavioral abnormalities.

5.5 CLINICAL EVIDENCE

VD deficiency is associated with several psychiatric and neurological disorders. For example, there is evidence for a relationship between low VD levels and anxiety and depression in fibromyalgia (Armstrong et al., 2006), whereas low VD levels were also observed in psychiatric patients with depression (Jorde et al., 2006; Schneider et al., 2000). Genetic variance in the VDR gene influences both cognitive functioning and depressive symptoms in older adults (Kuningas et al., 2007). VD deficiency is considered as a possible contributor to seasonal affective disorder (SAD), since SAD is more prevalent in winter time, when sunlight is reduced (Lansdowne and Provost, 1998; Rosenthal et al., 1984; Schlager, Schwartz, and Bromet, 1993; Spoont, Depue, and Krauss, 1991; Stumpf and Privette, 1989). Several studies have showed an association between low VD and mood disorders in older adults (Dumville et al., 2006), which are accompanied with impaired cognitive functions (Wilkins et al., 2006). A possible link between UVB light and autism has been discussed (Cannell and Hollis, 2008), as the disorder is less frequent in sunnier latitudes. Neuroprotective and immunomodulatory effects have a potential value in neurological and neurodegenerative disorders (Garcion et al., 2002). Low levels of calcidiol in pregnant mothers has been associated with an increased risk of schizophrenia of their children (McGrath et al., 2003). The morphological brain changes observed in DVD rats (see above) are consistent with the changes observed in human brains with schizophrenia (Harrison, 1999; Mackay-Sim et al., 2004; Schneider et al., 2000). Low VD status is also observed in patients with Alzheimer's disease (AD), schizophrenia and in elderly subjects with cognitive dysfunctions (Przybelski and Binkley, 2007; Wilkins et al., 2006). VD supplementation may prevent the development of multiple sclerosis (MS) (Brown, 2006; Hayes, 2000; Munger et al., 2004), of which VD-dependent rickets type I could be a risk factor for this disease (Torkildsen et al., 2008). The geographic incidence of MS indicates an increase in MS with a decrease in

sunlight exposure (Kragt et al., 2008; Raghuwanshi, Joshi, and Christakos, 2008; Torkildsen et al., 2008). Moreover, VD reduces the incidence of seizures (Christiansen, Rodbro, and Sjo, 1974). The potential role of VD in epilepsy is discussed above.

Poor VD intake resulting in osteomalacia was associated with bilateral cochlear deafness and episodic vertigo. A correlation was found between hearing impairment and muscular weakness, but daily consumption of calciferol improved unilateral hearing (Brookes and Morrison, 1981; Brookes, 1983; Brookes, 1985; Davies, Kane, and Valentine, 1984). Sensorineural hearing loss has been reported in patients with renal failure (Ikeda et al., 1987), although in these studies the authors postulated that hearing loss accompanied by VD deficiency is attributed to disrupted Ca^{++} concentration. Normacalcemic VDR KO mice demonstrated sensorineural hearing loss (paper III), however, suggesting that the VD/VDR endocrine system may be important in hearing function. There are also several risk factors for poor balance, such as muscle strength and low 25OHD levels (Bischoff-Ferrari et al., 2006; Inderjeeth et al., 2007). VD is required during aging of the musculoskeletal system, to prevent falls in older people (Gallagher, 2004; Montero-Odasso and Duque, 2005; Snijder et al., 2006).

Finally, VD appears to be involved in modulation of the brain neurotransmitters acetylcholine and catecholamine, which are known to be involved in the regulation of emotional behavior (Sonnenberg et al., 1986).

5.6 KNOCKOUT MOUSE MODELS TO STUDY VITAMIN D/VDR FUNCTION

The development of mutant/knockout models for both the VDR and 1α-OH provides powerful tools to delineate the distinct and indirect biological role of VDR and its ligand in several processes (Sutton and MacDonald, 2003). These provided mouse models of the inherited human disorders, VD dependent rickets type I and II (Beer et al., 1981; Fraser et al., 1973).

Several VDR KO mouse strains are currently known in the literature:

1) “Tokyo” mice, generated by disrupting the VDR gene in exon 2 of the first Zn finger motif of the DNA- binding domain (Yoshizawa et al., 1997);
2) “Boston” mice were generated by deleting a 5-kb section of the exon 3 fragment of genomic DNA, which encodes the second Zn finger of the DNA–binding domain (Li et al., 1997);
3) “Munich” VDR KO mice were generated by the disruption of the second exon and second intron of the first Zn finger (Erben et al., 2002); and finally,
4) “Leuven” VDR KO mice were generated by deleting a fragment encompassing exons 1 and 2 of the first Zn finger (Van Cromphaut et al., 2001) .

However, all these mice are considered to be VDR KO, despite the presence of a ligand-binding domain, as VDR KO mice retain the capability of producing a ligand-binding part, but cannot activate gene transcription (Bula et al., 2005). VDR KO mice of all strains were phenotypically normal at birth. Around weaning, they developed hypocalcaemia, secondary hyperparathyroidism and hypophosphatemia. At this age, VDR KO mice also became growth retarded and developed severe rickets and osteomalacia (Erben et al., 2002; Li et al., 1997; Van Cromphaut et al., 2001; Yoshizawa et al., 1997). Serum calcitriol levels were increased due to secondary hyperparathyroidism and they had increased renal

1 alpha-hydroxylase activity and decreased 24-hydroxylase activity. On the other hand, $1,25(OH)_2$ D levels were undetectable in 1α-OH KO mice, whilst 25(OH)D levels were elevated. Normalizing mineral levels by using a special Ca^{++} rich diet rescued the VDR KO abnormal phenotype. Treatment with a special diet consisting of 2.0% Ca^{++}, 1,25% phosphorus and 20% lactose with 2,2 IU VD/g was able to reverse rickets, hypocalcaemia, hypophosphatemia, and restore PTH levels (Bouillon, Van Cromphaut, and Carmeliet, 2003; Erben et al., 2002; Li et al., 1997; Li, Pirro, and Demay, 1998; Van Cromphaut et al., 2001). These findings confirm previous observations in humans (Hochberg, Tiosano, and Even, 1992; Kitanaka et al., 1998).

VDR KO mice showed alopecia and additionally, VDR KO female mice had defective reproductive organs and high mortality (Erben et al., 2002; Kato et al., 1999; Li et al., 1997; Van Cromphaut et al., 2001; Yoshizawa et al., 1997). Not all VDR KO phenotype disturbances were corrected by applying a special diet, as alopecia was still evident (Li, Pirro, and Demay, 1998).

As already mentioned above, VDR KO mice developed hypocalcaemia which occurs due to decreased intestinal calcium absorption (Song, Kato, and Fleet, 2003; Van Cromphaut et al., 2001). The decrease in intestinal calcium absorption was associated with low expression levels of mRNA TRPV6 (Van Cromphaut et al., 2001). CaBP-9k mRNA and protein levels were reduced (Bolt et al., 2005; Li, Pirro, and Demay, 1998; Van Cromphaut et al., 2003) and renal calcium reabsorption was also disturbed in VDR KO mice, whilst the normacalcemic VDR mutants showed an increase in their calcium/creatinine ratio (Erben et al., 2002; Li et al., 2001). Expression of renal CaBP-9k was decreased in both VDR and 1α-OH KO mice, but CaBP-28k mRNA levels were non-significantly decreased in VDR KO mice (Li et al., 2001), however significantly decreased in 1α-OH KO mice. A similar pattern was observed with TRPV5, which was normal in VDR KO mice, but decreased in 1α-OH KO mice (Hoenderop et al., 2002). A rescue diet lowered CaBP-9k expression in WT and both VDR and 1α-OH KO mice (Hoenderop et al., 2002; Song, Kato, and Fleet, 2003; Van Cromphaut et al., 2001). Normalization of $1,25(OH)_2D$ level in 1α-OH KO mice lead to increased expression of renal calcium transport proteins and normalization of calcium levels (Hoenderop et al., 2002). These data suggest that CaBP-9k may be an important component in the regulation of renal calcium reabsorption. VDR action is required for adequate calcium reabsorption. The intestinal VDR action and renal reabsorption are important for bone homeostasis (Bouillon et al., 2008). The growth plate abnormality has been observed in normacalcemic 1α-OH KO or double 1α-OH KO x VDR KO mice on a rescue diet and in VDR/RXR double KO mice (Panda et al., 2004; Yagishita et al., 2001). The number of bone marrow osteogenic progenitors decreased in normacalcemic aging VDR or 1α-OH KO mice, accompanied by reduced trabecular bone volume, suggesting that VD/VDR action in bone becomes critical with older age (Panda et al., 2004).

VDR KO mice also developed alopecia when circulating 25(OH)D and $1,25(OH)_2D$ levels were undetectable (Sakai, Kishimoto, and Demay, 2001). The absence of alopecia in WT mice with low circulating levels of VD metabolites suggests that the absence of receptors and ligands have different effects on hair follicles. The absence of alopecia in 1α-OH KO mice (Panda et al., 2001b) also confirms the effects of VDR on the follicle do not require ligand. VDR expression rescued alopecia, but not abnormalities in mineral ion or skeletal homeostasis in VDR KO mice (Chen, Sakai, and Demay, 2001; Skorija et al., 2005). Alopecia occurs in VDR KO mice due to impaired VDR action in keratinocytes of the hair follicle.

Mutations in the nuclear receptor co-repressor Hairless which binds VDR, resulting in transcriptional repression (Hsieh et al., 2003; Xie et al., 2006) are also associated with alopecia in mice (Miller et al., 2001). Dermal cysts and an increase in sebaceous glands were observed in VDR KO mice and hairless mice, similar to mice expressing a keratinocyte-specific Lef1 transgene with a mutation preventing its interactions with ß-catenin (Merrill et al., 2001). The absence of VDR impairs canonical Wnt signaling in keratinocytes and leads to the development of alopecia, due to a defect in keratinocyte stem cells (Cianferotti et al., 2007), suggesting ligand-independent effects of VDR are required for normal keratinocyte stem cell function. Another study showed 1α-OH is essential for normal epidermal differentiation, most likely by producing the VD metabolite, $1,25(OH)_2D$. These are responsible for inducing proteins that regulate calcium levels in the epidermis, which are critical for the generation and maintenance of the barrier (Bikle et al., 2004).

The skin of normacalcemic Boston VDR KO mice is hypersensitive to tumorigenesis in response to oral administration of a carcinogen. However spontaneous cutaneous tumors were rarely detected in VDR KO mice (Zinser, Sundberg, and Welsh, 2002). Optimal VDR signaling may contribute to the suppression of tumorigenesis, as VDR KO mice had reduced lung metastatic cancer growths (Nakagawa et al., 2004).

VDR KO mice fed a normal diet revealed defects in T cell proliferation and macrophage function, which was remedied through a rescue diet (Mathieu et al., 2001). The phenotype of dendritic cells (DC) in normacalcemic VDR KO mice also found some abnormalities (Griffin et al., 2001). VDR KO mice showed resistance to experimentally induced airway inflammation (Wittke et al., 2004) and parasitic *Leshmania* infection (Ehrchen et al., 2007).

VDR KO mice exhibit increased renal renin mRNA levels even after normalization of calcium homeostasis through a rescue diet (Li et al., 2002). This leads to high plasma Ag II levels, systematic hypertension and results in cardiac hypertrophy (Xiang et al., 2005). VDR KO mice developed cardiac hypertrophy and fibrosis (Simpson, Hershey, and Nibbelink, 2007) and different strains also showed uterine hypoplasia and impaired ovarian folliculogenesis (Kovacs et al., 2005; Yoshizawa et al., 1997). However, on a high calcium/lactose diet reproduction was normalized (Johnson and DeLuca, 2001), implying that hypocalcaemia is the major driving factor for reduced fertility.

The positive and negative effects of “rescue” diets for normalizing disturbed functions induced by VDR KO genetic dysfunction are summarized in Table 1. Results reported in this are based on our studies and other’s observations: (Amling et al., 1999; Bikle et al., 2006; Bolt et al., 2005; Bouillon, Van Cromphaut, and Carmeliet, 2003; Burne et al., 2005; Ellison, Eckert, and MacDonald, 2007; Erben et al., 2002; Hsieh et al., 2003; Johnson and DeLuca, 2001; Kalueff, 2005; Kalueff et al., 2006b; Kalueff et al., 2004a; Kalueff et al., 2004b; Kalueff et al., 2004c; Kalueff et al., 2005; Li et al., 2002; Li et al., 1997; Li, Pirro, and Demay, 1998; Meindl et al., 2005; Schlumbohm and Harmeyer, 2004; Simpson, Hershey, and Nibbelink, 2007; Song, Kato, and Fleet, 2003; Van Cromphaut et al., 2001; Xiang et al., 2005; Xie et al., 2006; Yagishita et al., 2001; Zinser, Packman, and Welsh, 2002; Zou et al., 2008).

Table 1. Effect of "rescue" diet on the phenotype of VDR mutant mice

Effects	**Positive**	**References**
Reduced body weight	Appling diet restores normal body weight partially, VDR KO males and females remained small even with special diet	Erben et al 2002; Song et al 2002; Burne et al 2005
Short lifespan	VDR KO mice live longer than 1 year	Yoshizawa et el 1997; Erben et al 2002
Impaired serum chemistries	serum Ca++, phosphorus, PTH, 1,25$(OH)_2$D level was normalized	Cromphaut et al 2001; Song et al 2002; Erben et al 2002
Hyperparathyroidism	Normalized blood calcium level prevents the development of secondary hyperparathyroidism and parathyroid hyperplasia (parathyroid cell proliferation)	Li et al 1998
Decreased intestinal calcium absorption	Increase intestinal calcium absorption, but expression of claudin 2 and claudin 12 was decreased in VDR KO mice in the intestine	Song et al 2002; Cromphaut et al 2001; Fujita et al 2008
Low expression of CaBP	Normalized renal calbindin D28 k; Normalized intestinal calbindin 9D k mRNA	Erben et al 2002; Li et al 1998; Cromphaut et al 2001
Impaired bone formation and mineralization	Normalize femur length; Restore bone mineralization, increase osteoblast number, prevents growth plate abnormalities, maintain normal growth plate	Song et al 2002; Li et al 1998; Amling et al 1999
Reduced fertility in males and females	" Rescue " diet corrected reproductive organs in both males and females: prevent uterine hypoplasia, improve folliculogenesis and spermatogenesis	Erben et al 2002; Johnson et al 2001
	No effect of the "rescue" diet	
Alopecia	VDR may regulate hair follicle cycling and in ligand-dependent manner. VDR action is independent of 1,25$(OH)_2$D in keratinocytes. VDR and hairless corepressor exists in the skin and drive the progression of the hair cycle and keratinocytes	Bikle et al 2006; Meindl et al 2005; Ellison et al 2007; Xie et al 2006; Hsieh et al 2003
Renal tubular calcium reabsorption	Urinary calcium/creatinine ratio was higher in VDR KO mice, VDR action is required for adequate calcium reabsorption in the kidney, by regulating the expression TRPV5 expression	Erben et al 2002; Hoenderop et al 2003
Chondrocyte cell proliferation	VDR-RXR are essential for normal development of growth plate chondrocytes	Yagishita et al 2001
Mammary gland morphogenesis	VDR KO fed with rescue diet developed enhanced mammary gland growth, ductal outgrowth, and enhanced branch points. Vitamin D signaling pathway participates in negative growth regulation of mammary gland	Zinser et al 2002
Skeletal muscle development	VDR is essential in muscle development, it is important for striated muscle	Endo et al 2003

Table 1 cont.

Expression of CaBP	No influence on renal calbindin 9D k, and brain calbindin 9D k. But functional VDR is required to suppress dietary-induced calbindin 9D k, but not renal calbindin D28 k. Epithelial calcium channel expression not changed	Erben et al 2002; Li et al 1998; Bolt et al 2005; Cromphaut et al 2001
Heart size and blood pressure	VDR negatively regulates the expression of renin, allowing for decreased angiotensin production and lower blood pressure. VDR KO mice exhibited profound cardiac hypertrophy	Li et al 2002; Xiang et al 2005; Simpson et al 2007
Behavioral dysfunctions	VDR KO mice fed with rescue diet demonstrated anxious profile	Kaluef et al 2004; Kalueff et al 2004; Kalueff et al 2006
Hearing loss	VDR KO mice fed with rescue diet exhibited sensorineural hearing loss	Zou et al 2008 (paper III)
Seizure threshold	VDR KO mice had shorter latency of convulsion	Kalueff et al 2006 (paper II)
Motor functions	VDR KO mice demonstrated abnormal swimming and impaired motor functions	Kalueff et al 2004; Burne et al 2004, 2005

The phenotype of 1α-OH KO mice is similar to that of VDR KO mice, and includes hypocalcaemia, hyperparathyroidism, growth retardation and osteomalacia, undetectable $1,25(OH)_2D$, but normal serum levels of 25(OH)D, and circulating $24,25(OH)_2D$ (Dardenne et al., 2001; Panda et al., 2004), similar to patients with pseudo vitamin D deficiency rickets (PDDR) (Delvin et al., 1981). PDDR patients, unlike patients with hereditary VD-resistant rickets and the mouse model KO (described above), do not exhibit alopecia. However, the ricketic phenotype of 1 α-OH KO mice can be reversed with the same "rescue" diet, which was applied to rescue VDR KO mice. At the age of three weeks old, application of "rescue" diet revealed hypocalcaemia, hypophosphatemia and secondary hyperparathyroidism, and the animals gained weight (Dardenne et al., 2004; Dardenne et al., 2003), although bone growth and femur size remained significantly small. For optimal osteoblastogenesis and osteoclastogenesis, $1,25(OH)_2D$ is needed, as shown by the sufficient effects of $1,25(OH)_2D$ treatment for femur growth in 1 α-OH KO mice (Dardenne et al., 2003; Panda et al., 2004).

5.6.1 Behavioral effects of VD

VDR KO mice on a normal calcium diet display a variety of subtle behavioral abnormalities such as abnormal grooming patterns (higher grooming frequency and duration, with abnormal organization of grooming and increased interruption) (Kalueff et al., 2004c; Kalueff et al., 2005), a sign of high anxiety. Additionally, VDR KO mice display impaired motor performance (Burne et al., 2005; Kalueff et al., 2004a) and increased anxiety (Kalueff et al., 2004b).

VDR KO mice showed anxious phenotype such as neophobic response to novel food (paper IV), but had normal olfactory and gustatory functions, normal spatial memory and an anhedonic phenotype (Burne et al., 2005; Kalueff, 2005; Kalueff et al., 2006b; Minasyan et al., 2007, paper IV) . Moreover, VDR KO mice displayed abnormal social

behavior, reduced barbering and aggressiveness. Impaired nest-building activity, such as less paper damage and the building of less complete nests, could be associated with altered prolactin secretion in VDR KO mice (Keisala et al., 2007). Burne et al (Burne et al., 2006; Burne et al., 2005) studied VDR KO mice and showed that they were able to swim 1m of a laneway, and reach the visible platform, but had sinking episodes. This group also showed that VDR KO mice had a normal acoustic startle response, but impaired prepulse inhibition. Hypolocomotion in several tests and short stays on a rotating rod suggested impairment in muscular and motor coordination, rather than emotional behavior.

However, as noted by A. V. Kalueff (Kalueff, 2005), one remarkable difference of the VDR KO mouse strain used in Burne`s laboratory was that they were hypocalcemic, as they were raised on normal calcium and therefore developed hypocalcaemia and growth delay (Burne et al., 2006; Burne et al., 2005). Hypocalcaemia could be due to consumption of water supplemented with 2mM Ca^{++}, whereas the “rescue” diet is only able to normalize serum Ca^{++} levels (Amling et al., 1999; Bouillon, Van Cromphaut, and Carmeliet, 2003; Erben et al., 2002; Li et al., 1998; Li, Pirro, and Demay, 1998; Van Cromphaut et al., 2001). *In vitro* studies, especially muscle phenotyping of VDR KO mice, suggest a direct effect of the VDR-VD endocrine system on the later stages of muscle cell development (Endo et al., 2003).

Comparing behavioral phenotypes of DVD-deficient rats (discussed in section 5.4) to VDR KO mice, the difference is most likely due to the absence of the ligand, VD, and not dysfunctional receptors. DVD-deficient rodents present possible gene–environment interactions, which are absent in VDR KO mice. This is the reason to assess several different parameters when interpretating emotional behavior of rodents (Crawley, 2000; Crawley, 1999) .

6 AIMS OF THE STUDY

The aim of this study was to characterize the neurological phenotype of VDR KO mice.
To understand the role of VDR and 1,25$(OH)_2$D in the regulation of physiological functions of the central nervous system, the following tasks were formulated:

I. To examine the behavioral response to novel food, to add more understanding of the functions of VDR in emotional behavior. To study cognitive and sensory functions in VDR KO mice;

II. To study the role of 1,25$(OH)_2$D in the regulation of seizure activity and the possibility of its anticonvulsant role;

III. To examine the role of VDR in the regulation of seizure activity;

IV. To assess hearing in mice with a mutated VDR gene;

V. To assess the balance functions of VDR KO and 1α-OH KO mice.

7 MATERIALS AND METHODS

7.1 ANIMALS

The VDR KO mice were initially generated at the University of Tokyo (Japan) (Yoshizawa et al., 1997). The mutation was also transferred in our laboratory (by approximately 10 backcrosses) to an NMRI background strain (Fig. 4). All mice used were littermates of 129S1/SvImJ (129S1) and NMRI background strains produced by heterozygous (HZ) crosses. 1α-OH KO mice were provided by prof. Dardenne (Dardenne et al., 2001) and produced by littermate HZ crosses on a C57BL/6 background strain (Fig. 5). To normalize the blood mineral ion levels, all VDR KO and 1α-OH KO animals received a special "rescue" diet containing Ca 2%, 1,25 % P, and 20 % lactose (Lactamin AB, Sweden).

Genotyping of mice was confirmed by PCR on DNA prepared from tail tissue. For VDR KO mice, four primers were used to amplify a 166 bp VDR (forward, 5'-CTG CTC TTC TTA CAG GGA TGG-3'and reverse, 5'-GAC TCA CCT GAA GAA ACC CTT G-3') and 400 bp Neo (forward, 5'-ATC TTC TGT CAT CTC ACC TTG C-3' and reverse, 5'-CAA GCT CTT CAG CAA TAT CAC G-3') band from the target allele. 1α-OH KO genotype was confirmed using 2 primers (forward 5'-GTC CCA GAC AGA GAC ATC CGT-3' and reverse 5'-GCA CCT GGC TCA GGT AGC TCT TC-3').

Adult KO and WT mice were used for experiments. The animals were experimentally naïve and housed individually or 2-4 per cage (width 20,7 cm, length 36,5 cm). All animals were kept in a 12/12-hour light/dark cycle in a standard virus/parasite-free facility (temperature, 22±2° C; humidity, 55±5%) with free access to tap water and pellets ad libitum (except in the food finding experiments). The use of all animals in experiments reported here was approved by the Ethical Committee of the University of Tampere. Animal care and experimental procedures were conducted in accordance with European legislation.

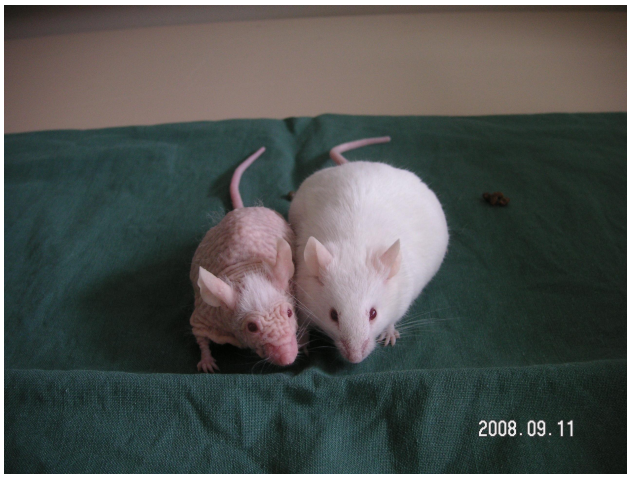

Figure 4. Phenotype of VDR KO (left) vs. WT NMRI mice at a 12-month age

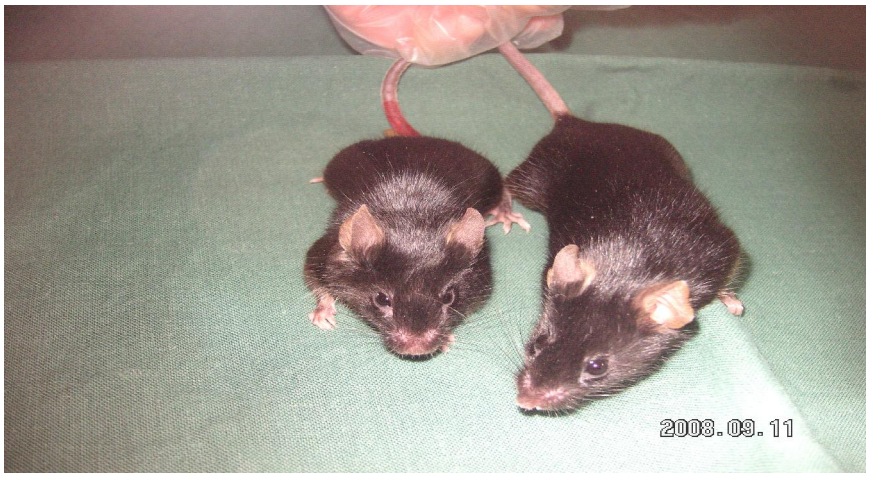

Figure 5. Phenotype 1 α-OH knockout mice vs. WT at a 5.5-month age

7.2 EQUIPMENT AND PROCEDURES

The detailed experimental procedures are presented in the articles included in this thesis. Table 2 shows all implemented procedures.

Table 2. Methods used in the original publications

Methods	Articles
Y-maze spatial memory and exploration	IV
Y-maze familiar and novel food test	IV
Buried food test	IV
Two-bottle preference test	IV
Hedonic responses test	IV
Genotyping	II,III,IV,V
PTZ injection	I,II
Vitamin D injection	I
Ca++ plasma level measurement	I,II,III,V
Balance function tests	V
ABR measurement	III
Plastic embedding	III
Immunohistochemistry	III,IV
Light microscope	III
Immunofluorescent confocal microscopy	III,V
Phase contrast microscopy	III, V
Statistical analysis	I, II, III, IV, V

The novel food test was carried out using different food (IV). Emotionality was assessed using different unfamiliar food (bread, vanilla and onion) and based on the suppression of

spontaneous behavior in rodents by their natural aversion to novelty. Food was placed in one arm of a Y-maze and the mouse was able to find the food in both of two trials. All recordings were made for 5 min. The latency to eat (s) and number of times the mouse investigated the arm with and without food were used as measures of behavioral responses to novel food. All animals were allowed to explore the environment (without food) prior to testing, for 10 min to minimize the novelty factor and make mice more familiar with the environment.

The Y-maze (Fig. 6) test was applied to assess the animal spatial memory (Dellu et al., 2000). The test included two trials, with 30 min between trials. In the first trial, one of the three arms was closed and animals had access to two open arms. During the second trial, all three arms were open. For 5 min, the number and duration of each arm visit were recorded for both trials (IV).

The buried food pellet test (Nathan et al., 2004) was used to assess olfactory function. Mice were given a food-restricted diet for one day. On the test day, a familiar food pellet was covered under bedding material about 0.5 cm deep. The delay in finding buried food was taken as an index of olfactory abilities within a 5-min cut off time. The food was considered found if the mouse was holding the pellet in its forepaws. Bedding material was changed between animals (IV).

Figure 6. The Y-maze consisted of three identical arms with an angle of 120° between two arms. Each arm was 9x30x15 and made of Plexiglas. The height from the floor is 70cm.

The two-bottle test (Kalueff, Gallagher, and Murphy, 2006) was applied to study mouse gustatory functions and their baseline hedonic responses. Animals were placed individually and given a free choice between two bottles (one with a tasty solution and another with tap water). The solutions used in the study included: sweet (1% sucrose,

BDH Laboratory Supplies, England; 7 days), sour (50mM citric acid 1-hydrate, Riedel-de Haen A.G., Germany), bitter (100 M cycloheximide, Sigma–Aldrich, Germany), and salty (0.3M NaCl, J.T. Baker, Holland) for 4 days each. The position of bottles was switched every 24 h to avoid side preferences. The consumption of water and tasty solution was estimated simultaneously in both genotypes by weighing the bottles daily and calculating daily preference (%) as total taste solution/total water intake×100%. No food or water deprivation was used in this experiment (IV).

The testing of balance function was carried out using several tests: rotarod, tilting box, rotating tube. The swimming test was also used to evaluate the vestibular functions of mice (V).

Figure 7. Accelerating rotarod. Mouse placed on rotarod able to move forward, keeping itself on the rotarod when speed increases.

The rotarod (Fig. 7) was a PVC plastic rod with a diameter of 3.2 cm and length of 7 cm, elevated 22.5 cm above the ground. The surface of the rod was slightly roughened to minimize accidental slips. The mouse was placed vertically, side-oriented on the rod at a slow 4 rpm rotational speed. Speed gradually increased over the 120-s test session, up to 28 rpm. The rotarod apparatus was placed inside a dark box, equipped with an infrared video camera recording system. The camera (PCTV) was attached to a computer for data acquiring and processing. The animal was observed through a computer, and the rotarod was stopped when the mouse fell down, to obtain the latency to fall. The experiment consisted of three trials in a single day.

The tilting box (Fig. 8) was constructed with 6 mm thick Plexiglas, with a separate glass floor to prevent scratching the surface. The length of the box was 20 cm; the height and depth were 10 cm. The speed of tilting was 13.9°/s. The mouse was placed at one end of the box oriented by its nose. In some cases, the animal turned to the tail direction down slope. The light transparent plastic “wall” separated the box into two halves, preventing the animal from turning back before the box was tilted. The test was performed in darkness and recorded with a video camera, as mentioned above. The angle of falling down and latency to fall were measured during the test from the video recording. The angle of falling is the angle at which the forepaws/hind paws began slide to the opposite side, at which

point the test was ended. The ability for the animal to retain its posture whilst falling down, as well as landing on all four paws, was also recorded.

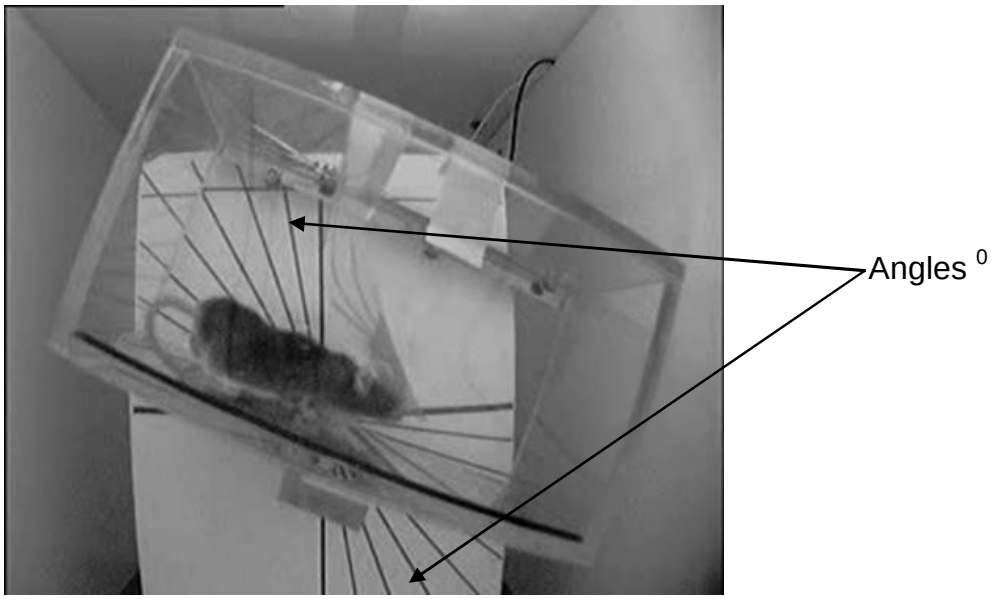

Figure 8. Tilting box in action with the mouse inside.

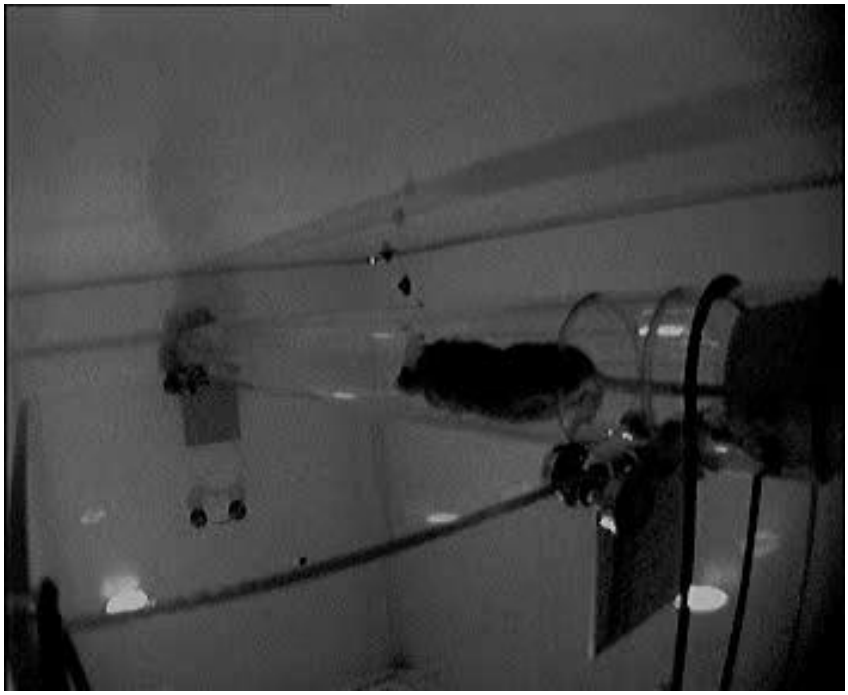

Figure 9. Rotating tube equipment. The mouse is able to move forward and backward inside of the rotating tube

Fig. 9 shows a Plexiglas tube (length: 44,5 cm), where the mouse was placed into the tube. Three different diameters (24, 34 and 41 mm) were used according to the weight and size of the mouse. The mouse was placed inside the tube and the ends of all the tubes

were blocked to prevent escape. The tube turned with a speed of 35 rpm. The test was conducted in darkness and under video control. The following measurements were recorded: (1) angle of rotation: 45° or 90°; and (2) capability of mouse to reposition itself during rotation. Between subjects, each apparatus was thoroughly cleaned (using wet and dry cloths), to remove odour stimuli and avoid false negative results.

All mice tested in the balance test were also investigated in a swim test, as reported earlier (Kalueff et al., 2004a). Briefly, glass cylinders with diameters of 17 or 21 cm were filled with warm water (22°C-25°C) to a height of 20 cm from the top. The mouse was placed on the water surface and its swimming performance recorded for 3 min. Normal swimming behavior was recorded as the animal resurfacing (in a horizontal position), keeping its nose above the surface of the water and pursuing toward the wall of the cylinder. Abnormal swimming was considered to be when the animal swam vertically with its head up and most of the animals appeared to sink (the animal's head dipping below the surface). Abnormally swimming animals were removed from the water immediately. The water was changed between the animals.

Pentylenetetrazole (PTZ) was used to induce chemical seizures in mice. Each animal received an i.p. injection of PTZ (70 mg/kg, Sigma, UK). Seizures and seizure latency times were observed visually over a 15-min period. The latencies of the first twitch, Straub tail, oro-facial, clonic and tonic seizures were analyzed. The total observation time was 900 s in mice not showing the respective behaviors. Mortality in both groups was assessed over a 30-min period. An animal was considered dead if the heart was not beating upon manual checking (the latency of death was counted as 1800 s if the animal remained alive after a 30-min observation period). Seizure severity was scored according to Racine (Racine, 1972): 0 (no response), 1 (freezing), 2 (head nodding or isolated twitches), 3 (oro-facial seizure), 4 (clonic seizure), 5 (tonic seizure), 6 (death). Clonic seizures consisted of rhythmic contractions of forelimb and/or hind-limbs. Tonic seizures consisted of a rigid extension of the fore- and/or hind-limbs with or without posture loss (I, II).

To study the anticonvulsant role of VD in PTZ-induced seizures, 33 µg/20 µl of $1,25(OH)_2D$ (Leo Pharma, Denmark) in 4 nM solution in 20% isopropanol was injected subcutaneously 40 min prior to induced seizures. To assess the time-course of anticonvulsant effects, the same compound was injected 40 min, 3, 6, 12, and 24 h prior to PTZ-induced seizures. Control mice received 20% isopropanol (I).

For measuring hearing in mice, the auditory brain stem responses (ABR) were used. The ABR test is reliable, objective, non-invasive and painless. Brain wave activity in the auditory centres of the brain was recorded in response to a series of sound clicks presented to each ear. The ABR test indirectly estimates the level of hearing in the peripheral auditory system (middle ear and inner ear). BioSig32 (Tucker Davis Technologies, USA) was used for ABR threshold recordings in mice under anaesthesia with Domitor (0.8 mg/kg medetomidine hydrochloride) and Ketalar (80 mg/kg ketamine hydrochloride). Click durations of 50 µs and a repetition rate of 21.1/s were used for stimulation. Responses from 512 sweeps were averaged with a gain of 20 at each intensity level using a filter of 0.1–3 kHz. Thresholds were judged by visible repeated responses (III).

Plastic embedding was used to analyze the morphology of cochlea. 2- µm sections of cochlea were prepared with a microtone section machine (LEICA RM2265, Germany) and

slides stained with toluidine blue for light microscopy. Condensed nuclear staining with toluidine blue of spiral ganglion cells is a sign of degeneration (III).

To study morphology of vestibular organ, mice were sacrificed with CO_2 and after perfusion with phosphate buffered saline (PBS) (pH 7.4) the saccule, utricle and ampulla were isolated from the vestibulum under stereomicroscope. They were then incubated in FITC-labelled phalloidin (50 µg/ml; Sigma-Aldrich, USA) for 40 min and DAPI (Sigma-Aldrich, USA) for 10 min. The saccular macula and utricular macula were carefully dissected and mounted with Gel Mount™ Aqueous Mounting Medium (Sigma-Aldrich, USA). The morphology of the vestibular organ in WT and VDR KO mice was demonstrated with phase contrast microscopy (V). Light microscopy was used to evaluate the cochlear sections stained with toluidine blue (III).

Immunohistochemistry (IHC) was applied to determine the presence and tissue distribution of various proteins. The IHC technique allows visualization of antigens in paraffin-embedded tissues via sequential application of specific antibodies to antigen (primary antibody), followed by the enzymatic activation of chromogen resulting in a visible product at the antigen site. Negative and positive control samples and tissues were analyzed together within the same experiments in order to eliminate false positive or false negative results. Non-immune IgG, neutralization of the antibody with recombinant proteins prior to analysis, or an irrelevant antibody (not expressed in tissue) were used as negative controls. Tissue samples that are known to express the protein of interest served as positive controls. Antibodies used are presented in Table 3. Immunostaining was assessed using a semi-quantitative method. A scoring system was used to determine the intensity of staining (III, V).

Table 3. Antibodies used in Papers III and V

Antibody	Antibody type	Manufacturer
VDR	Rabbit polyclonal	Abcam, UK, ab12129
Synaptophysin	Mouse monoclonal	Sigma-Aldrich, USA
Connexin 26	Rabbit polyclonal	Zymed, USA
Kir4.1 (KCNJ10)	Rabbit polyclonal	Alomone Labs, Israel
TRPV 4/6,	Rabbit polyclonal	Alomone Labs
caspase 3	Rabbit polyclonal	R&D system, Inc., USA
IgG	Rabbit polyclonal	Invitrogen, USA

Immunofluorescent confocal microscopy was used to evaluate immunofluorescently stained slides and to identify the presence of VDR in different parts of the cochlea and vestibular organ (III, V). Expression of key genes in the regulation of cochlear functions, such as Connexin 26, KCNJ10, TRPV4/6 and caspase 3 (IV) were also evaluated.

Confocal microscopy was used to quantify caspase 3. A positive percentage was calculated on the basis of caspase 3 activation count and total nuclear DAPI nuclear staining (III). Under confocal microscopy sections of vestibular organ were studied. Sections were studied under an Olympus microscope IX70 with ANDOR IQ (FITC fluorescence at 488 nm with a laser beam; Alexa Fluor® 568 fluorescence at 568 nm with a laser beam; DAPI with a 340-380 nm filter).

Plasma Ca^{++} levels were analyzed in WT and VDR KO mice, and in WT mice with chemically induced seizures. Blood was taken from a heart puncture to measure plasma Ca^{++} and P^{++} levels by atomic absorption spectroscopy (Yhtyneet Laboratoriot, Helsinki, Finland).

7.3 Statistical analysis

Non-parametric Mann-Whitney U test was used for independent samples to analyse results in Articles I, II, IV, and V (data was analyzed by SPSS 11.5 software). One-way ANOVA test was also applied (I). Wilcoxon signed ranks test was used for two related samples (IV). Correlation between plasma Ca^{++} levels and seizure measures were analyzed using Spearman rank-order correlation coefficient (R) in article I. Article III analyzed data using the Student t test. Significance was accepted at 0.05 levels. Results are presented as MEAN ± S.E.M (I, II, III, and IV) and MEAN ± S.D (V).

8 RESULTS

All results of the studies are presented in the original papers (I-V) in detail. The statistical results are also described in these publications. Results published in reports I-V are summarized below.

8.1 AFFECTIVE AND COGNITIVE PHENOTYPES

8.1.1 Altered emotionality: food neophobia in Y-maze (IV)

The novel food (such as bread, vanilla and onion) versus familiar food (normal food pellets) evoked robust emotional responses in mice. Both WT and VDR mutants mice showed no genotype differences in the latency to eat bread in trial 1. After 2 h, in the second trial, WT showed a shorter latency than the KO group. Although the VDR KO mice showed a robust neophobic response to novel food (bread), the total exploratory activity (entries to arm with food reinforcement and empty arms without food) in both trials was similar for both genotypes. In the test with another unfamiliar food (vanilla), both groups had the same delay in eating the familiar pellet covered with vanilla powder. After 4 h in the second trial, VDR mutants started to consume food after a shorter time compared to WT mice, but the behavioral difference of VDR mutants became apparent through reduced exploratory activity on both trials. Interestingly, in the presence of onion, WT mice showed an aversive response and a longer latency to eat, while the VDR mutants showed a shorter latency to eat. Exploratory activity was higher in WT (compared to the VDR KO) mice. In all tests with novel food, for both genotypes the entries to the food arm were statistically significantly lower than those to the empty arms.

8.1.2 Normal spatial memory function (IV)

We further investigated memory function in VDR KO mice, in order to examine whether the difference in eating latency towards novel food and exploratory activity (mentioned above) were due to possible cognitive impairments in our mice. The spatial memory test for both genotypes appeared to be normal, as demonstrated by similar latency of entering the novel arm, and time spent exploring this arm.

8.1.3 Normal sensory functions (IV)

According to the novel food test results (difference in the latency of eating in both genotypes), it was important to study olfactory and gustatory functions. Both WT and mutant mice showed similar latencies of finding and eating food, serving as an index of normal olfactory functions. All mice of both groups were similar in the consumption of sweet, salt, sour and bitter solutions. Both groups consumed less of the salt solution, but liked citric acid and disliked the bitter taste.

8.1.4 Unaltered hedonic response (IV)

The study of baseline hedonic responses was necessary to show whether any genetic defect in VDR could evoke anhedonic depression. The depression domain was not studied previously in VDR KO mice. In this study we obtained a similar sucrose preference in both genotypes, showing the absence of anhedonia in this model. This finding supports the absence of notable depressive-like behaviours in VDR KO mice.

8.2 IMPAIRED MOTOR AND BALANCE FUNCTIONS (V)

In Article V, we investigated the vestibular functions in VDR KO mice on 129S1 and NMRI background strains. We also examined the balance functions of 1α-(OH) KO mice, in order to study the role of 1,25(OH)2D in the regulation of vestibular and motor functions.

8.2.1 Impaired balance functions in VDR knockout mice on a 129/S1 background

The Rotarod test in 12-month old VDR KO mice showed a shorter mean in the time it took to fall down than WT animals. The mean (average of all three trials) latency to fall down assessed the motor functions of the animals. A long latency is an index for good motor functions because it shows how animals are able to stay on a rotarod with accelerating speed, as was obtained in WT mice. The tilting box test found a statistical difference in vestibular performance between genotypes. Indicated by the angle of falling, 12-month old VDR mutants showed impaired balance and postural behavior. A lower angle when falling indicates the possibility of defects in retaining posture whilst falling.

8.2.2 Normal performance of VDR knockout mice on NMRI background strain in rotarod, tilting box and rotating tube

Unlike VDR mutants on a 129S1 genetic background strain, KO mice of the NMRI strain did not show any statistically significant impairment for balance and postural capabilities in the rotarod, tilting box or tube. Both genotypes showed similar mean latency to fall down. The angle of falling down was smaller in VDR mutants than in WT mice, but the difference was not statistically significant. Another test for balance and postural capability such as the rotating tube test did not show any statistical difference between genotypes in any measurement.

8.2.3 Impaired swimming in VDR knockout mice

Swimming was affected in VDR KO mice of both genetic background strains. Adult mutants showed vertical swimming and sinking, whereas adult or aged WT mice showed normal horizontal swimming without sinking (Fig. 10).

8.2.4 Unaltered balance functions in 1α-OH knockout mice

Motor functions, balance and posture were not affected by the targeted mutation of the 1 α-hydroxylase gene. Moreover, both genotypes demonstrated the same motor, balance and postural behavior in the Rotarod, tilting box, rotating tube and swimming tests (Fig. 11).

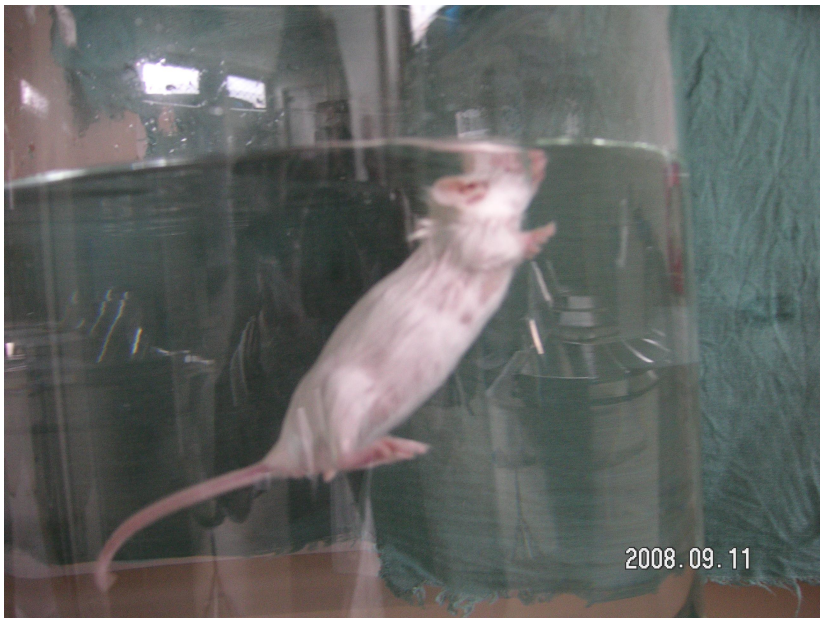

Figure 10. Abnormal swimming of a 5.5-month old NMRI VDR KO mouse

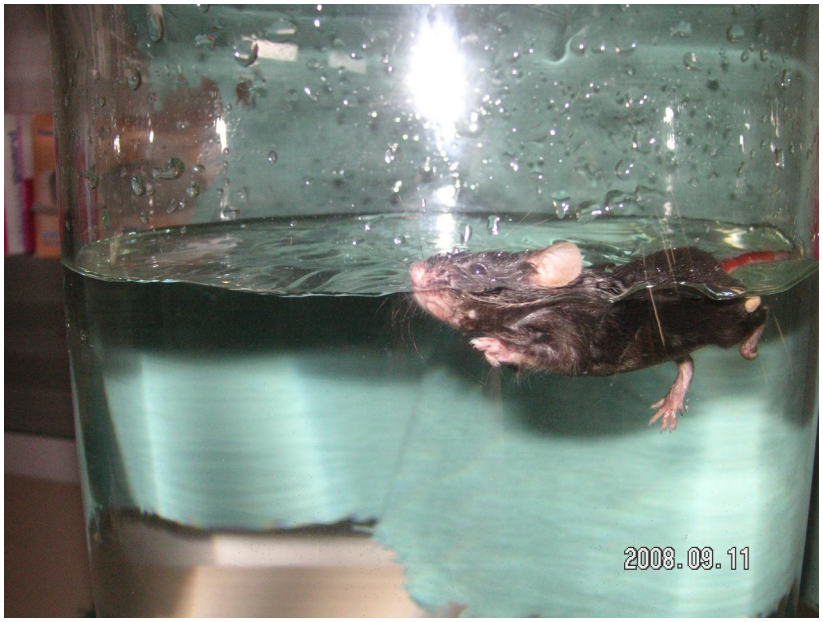

Figure. 11. Normal swimming of a 5.5-month old C57BL/6 1α-OH KO mouse

8.2.5 VDR expression in the mouse vestibular organ (V)

VDR were detected in the nuclei of the epithelium of the endothelial lining of crista ampullaris, membranous semicircular canal, and the surrounding osteocytes in WT mice.

8.2.6 Morphological study of the vestibular organ (V)

Saccular macula, utricular macula and crista ampullaris were morphologically normal in VDR KO mice. The crystal statolith of macula was formed in both genotypes.

8.3 INCREASED SENSITIVITY TO CHEMICALLY-INDUCED SEIZURES (I, II)

We used a convulsive doze of Pentylenetetrazole PTZ (70 mg/kg) because this dose does not show differences in seizure susceptibility for the different sexes (Medina, Manhaes, and Schmidt, 2001). Animals had seizures within 3 to 5 minutes. PTZ mostly produced generalized seizures in our mice. In most cases, the animals that developed tonic–clonic seizures died (Table 4).

Table 4. Effect of single administration of PTZ (70 mg/kg) on tonic-clonic convulsions and evoked mortality. In table presented wt vs. to VDR KO mice, and treated vs. to untreated mice. Mice were treated with $1,25(OH)_2D_3$, the dose 33 µg/20 µl in isopropanol. Tonic/clonic seizures are obtained in all groups.

Group	N of mice with Tonic/Clonic seizure (%)	N of dead mice (%)	Articles
WT 129S1	70/80	40	II
VDR mutants 129S1	100/100	90	II
NMRI non-treated	82/91	55	I
NMRI treated	45/73	18	I
NMRI x 129S1 non-treated	Number of mice with tonic/clonic seizures not available	91	I
NMRI x 129S1 treated (40 min)	Number of mice with tonic/clonic seizures not available	82	I

8.3.1 VDR mutant mice showed increased baseline seizure phenotype

Detailed results are shown in Article II. In this paper we studied whether VDR was involved in the regulation of seizure activity in VDR KO mice. VDR mutants showed a shorter latency to the first twitch, clonic seizure, tonic seizure and death, when compared with WT littermates. Mortality rate and Racine score were also significantly higher in the VDR KO mice (Table 5).

8.3.2 1,25 $(OH)_2D$-treated mice demonstrated a rapid decrease in seizure severity

In Article I we investigated the regulation of seizure activity by 1,25 $(OH)_2D$. We showed that a dose of 33 µg/20 µl was able to decrease seizure activity within a short time (40 min). As was shown in the earlier study (Siegel et al., 1984), in which intravenous injection of 1,25 $(OH)_2D$ had significant elevation of seizure threshold in rats, lasting within 30 min. The duration of clonic/tonic seizures, and total duration was shorter than in untreated controls. We also wanted to see if 1,25$(OH)_2D$ given at the same dose, but injected intraperitonialy, was able to decrease seizure severity at 40 min, 3h, 6h, 12h, and 24 h. This experiment used hybrid NMRI x 129S1 mice, in order to see the effect of 1,25$(OH)_2D$ on seizure activity in another genetic strain. 1,25$(OH)_2D$ produced a significant anticonvulsant effect at 40 min prior to PTZ administration. Latencies to tonic, clonic seizure and death increased, whereas treatment at 3 h, 6 h, 12 h and 24 h prior to PTZ administration, showed no significant effect on seizure activity (Table 5).

Table 5. WT mice compared to VDR mutant mice, drug-treated mice compared to non-treated mice. *, **, *** show difference for WT and non-treated animals

Group	Latency to the first twitch (s)	Latency to clonic seizures (s)	Latency to tonic seizure (s)	Latency to death (s)
WT 129S1 vs. VDR mutants 129S1	Significantly longer *	Significantly longer **	Significantly longer *	Significantly longer *
NMRI non-treated vs. NMRI treated	no difference	no difference	no difference	Significantly shorter ***
NMRI x 129S1 non-treated vs. NMRI x 129S1 treated	no difference	Significantly longer*	Significantly longer*	Significantly shorter*

8.4 ABNORMAL HEARING PHENOTYPE (III)

8.4.1 Progressive hearing loss (III)

ABR results showed that VDR KO mice developed earlier age-related hearing loss. Both WT and VDR KO mice showed age-related hearing loss. Young VDR KO mutants (under 6 months) had an elevated threshold and irregular waveforms (40 dB, except 1 wave) compared to young WT, which are able to hear with a threshold of 25-30 dB SPL. The difference between adult and aged WT and KO mice was not statistically significant, as both adult (6-12 months) and aged (>12 months) VDR KO mice demonstrated the same threshold of dB. The difference between young VDR KO and WT mice, and the lack of a statistical difference between young and adult mutants, indicates that VDR mutants developed an earlier impairment in their hearing.

8.4.2 Normal cochlear morphology (III)

Analyses of cochlear morphology between VDR mutants and WT mice did not show any difference in structures such as the organ of Corti, lateral wall and spiral ganglion cells. Mouse monoclonal antibodies for synaptophysin used under the inner and outer hair cells, detected the efferent nerve endings. Both WT and VDR KO mice had a normal layer of cells in the row of outer hair cells. Interestingly, both WT and VDR KO mice had reduced numbers of spiral ganglion cells, obtained using condensed nuclear staining with toluidine blue, which could be considered as a sign of degeneration.

8.4.3 Gene expression analyses in the cochlea (III)

We did not find VDR expression in VDR KO mice, which was robust in WT mice in different sections of cochlea (IV). We wanted to study the expression of genes involved in hearing loss. In order to study Connexin 26 disorder (a mutation in connexin 26 causes hearing loss), we studied the expression of the GJB2/Connexin 26 gene in cochlea. Connexin 26 was detected in the Corti organ, spiral ligament and capillary of the stria vascularis. Further analyses showed the expression of KCNJ10 and TRPV4 genes, which are responsible for expression of potassium and calcium channels. The expression of these proteins was not affected. Caspase 3 activation was analysed to study age-related hearing loss. Activation was detected in cochlear cells of both WT and mutants; however Caspase 3 activation was decreased in the cochlea of VDR KO compared to WT mice.

8.5 CALCIUM LEVEL (I, II, III, V)

We measured plasma calcium levels in the VDR KO mice in order to see how a calcium-rich diet normalizes Ca^{++} and Pi^{++} blood levels compared to WT mice. The measurement of Ca^{++} was needed to understand whether increased severity of seizures was due to hypocalcaemia (I, II). VDR KO mice showed slightly lower Ca^{++} levels (II) and Pi plasma levels were slightly higher in mutants than in WT controls (V).

9 DISCUSSION

Here we report that our findings indicate the importance of VD in the normal functioning of the central nervous system (I-V). Previous reports of VDR KO mice characterized several abnormal behavioral domains of these mice (Burne et al., 2006; Burne et al., 2005; Kalueff et al., 2006b; Kalueff et al., 2004a; Kalueff et al., 2004b; Kalueff et al., 2004c; Kalueff et al., 2005). Behavioral data from VDR KO mice and also VD deficient models of rodents indicate that VD is involved in the regulation of neurological functions (Almeras et al., 2007; Altemus et al., 1987; Armstrong et al., 2006; Becker et al., 2005; Burne et al., 2004a).

9.1 NONFUNCTIONAL VDR LEADS TO NEOPHOBIA

The term neophobia refers to a continued and abnormal fear response to anything new. In rodents, food neophobia can be assessed with novel food (Merali, Levac, and Anisman, 2003; Zhu et al., 2006), which evokes anxiety-related behavior. Anxiety levels can be measured from the latency to consume novel foods (Zhu et al., 2006). In our study (IV) we placed novel food in the Y-maze (Fig. 6). The Y maze test is a common and quite easy to handle test of two-trial recognition memory test in rodents (Dellu et al., 2000; Dellu et al., 1992). Since VDR KO and WT mice did not show any difference in their memory functions (IV), we did not focus on the cognitive abilities of mice in the novel food tests.

Investigation of empty and food arms was taken as a measurement of exploration. We found that unfamiliar food had effects on an emotional behaviour, which differed between WT and VDR KO mice. Results from the test with bread showed that both groups had similar latencies of eating food and the number of entries, whereas in the second trial mutant mice demonstrated a longer latency of eating than WT mice. In tests with vanilla and onion, VDR KO mice displayed reduced exploration and increased anxiety (Crawley, 2000; Crawley, 1999) This is consistent with the previously reported anxiety profile in our VDR KO mice (Kalueff et al., 2004b), and with altered locomotor activity in VD deficient rodents in different tests (Altemus et al., 1987; Becker et al., 2005; Harms et al., 2008).

Previous observations (unpublished data) also showed that daily food consumption (g) of adult WT male mice is statistically higher than in mutants (5,35 ± 0,28 vs. 3,78 ± 0,25; $p< 0,05$). Consequently, WT mice need a higher daily intake (kJ) than mutant mice (66,9 ± 3,51 vs. 51,4 ± 3,44; $p< 0,02$), which could explain the difference between latencies through different energy requirements and desire to eat. Experiment with familiar food pellets (same test conditions as described above) showed that both genotypes had a similar latency to eat, in which VDR KO mice displayed even increased exploratory activity (IV). The rescue diet used for VDR KO mice generally normalized their plasma Ca^{++} levels (Li et al., 1998), showing that decreased locomotor activity was not due to hypocalcaemia. VDR KO mice exhibited more “risk assessment” behaviors such as short latencies in the vanilla and onion tests. Such behaviors are interpreted as reflecting anxiety (Zhu et al., 2006).

Importantly, VDR are found in the limbic system (Langub et al., 2001; Walbert, Jirikowski, and Prufer, 2001), a brain structure known to be involved in the regulation of emotion. VD is also involved in the regulation of mediators regulating emotional behavior (Garcion et al., 2002). Developmental VD deficiency alters the behavior of rats, such as hyperlocomotion, which is not due to changes in stress responses (Eyles et al., 2006). Another study showed that maternal VD deficiency (DVD) reduces the number of dopamine cells in adult offspring (Cui et al.). As it is known that failure of dopaminergic neurotransmission results in movement disorders, this may be one of the reasons for the altered locomotor activity of DVD rodents. Given our results in VDR KO mice, we can suggest that VDR is involved in the regulation of emotional behavior. Recent pilot data from the University of Utrecht (Netherlands) strongly support the notion that VDR gene is implicated in neophobia. In this study, quantitative traits loci (QTL) analysis (Kas et al., 2008, unpublished data) in mice revealed that chromosomal locus of VDR gene is responsible for neophobic response to novelty.

9.2 INCREASED THRESHOLD LEVEL TO SEIZURES BY 1,25(OH)$_2$D AND INCREASED SEIZURE ACTIVITY IN VDR MUTANT MICE (I, II)

Chemically induced seizures in rodents are widely used as a model for epileptogenesis and represent human epilepsy, characterized by a high degree of clinical relevance. In our studies (I, II) we used pentylenetetrazole (PTZ) to produce seizures in mice. Results presented here show for the first time that the lack of VDR leads to more severe seizures in VDR KO mice (II) and that 1,25(OH)$_2$D is able to reduce the severity of convulsions when administered subcutaneously 40 min prior to PTZ (I). The mortality rate was also increased in VDR KO mice and decreased in mice treated with 1,25(OH)$_2$D.

The PTZ model has proven to be effective in producing seizures in rodents and for testing the efficacy of drugs in reducing convulsions (Gupta and Malhotra, 1997; Morgan et al., 2006; Mortazavi et al., 2005; Nehlig et al., 2006; Pourgholami et al., 1999; Woldbye, 1998). PTZ acts on the picrotoxin (PTX) site of the γ-aminobutyric acid (GABA) type A receptor, blocking GABA-mediated Cl- influx through interactions of the Cl channel, leading to depolarization of the neuronal membrane and the propagation and maintenance of seizure activity (Sieghart, 1995; Sieghart et al., 1999). There is a demonstrated sex difference in the response to intraperitoneally injected PTZ, but at a high dose such as 70 mg/kg, there is no sex difference in susceptibility to seizures (Medina, Manhaes, and Schmidt, 2001), which is why we applied this dose in our experiments (I, II). Since the mechanisms of actions of 1,25(OH)$_2$D on seizure activity are unclear, we discussed the possible reasons in our studies (I, II). For example, the role of GABA transmission in the regulation of seizures and how 1,25 (OH)$_2$D as a neurosteroid (Garcion et al., 2002) protects against GABA-A blockers via regulation of GABA-Aα4 gene expression (Feron et al., 2005), has been reported (Gale, 1992).

Likewise, 1,25(OH)$_2$D/ VDR play an important role in the regulation of seizure activity, as was shown in animal models of epilepsy (Siegel et al., 1984) (papers I and II). 1,25(OH)$_2$D given 40 min before the induction of seizures significantly reduced seizure activity and, as a consequence, the mortality of animals. Using a special diet we rescued our VDR KO mice, which normalized plasma levels of Ca^{++} and Pi^{++} (I, II, III, IV). Plasma Ca^{++} levels were unaltered in our experimental mice (I). These data suggest that the change in seizure control in our VDR KO mice and 1,25(OH)$_2$D -injected animals is not due to reduced levels

of Ca^{++}, or the calcemic effects of 1,25$(OH)_2$D. Moreover, our aged VDR mutants showed intracranial calcification (Kalueff et al., 2006a), due to hypocalcaemia and hyperparathyroidism (Friedman, Chiucchini, and Tucci, 1987; Moriwaki et al., 1985), whilst adult VDR KO animals (II) showed only slight hypocalcaemia.

Increased total duration of seizures, mortality rate, reduced latency to death and to seizures in VDR KO mice (II), suggest that the anticonvulsive functions of 1,25$(OH)_2$D (I) may be mediated through its nuclear receptors. These functions are very rapid for short-time effects (40 min), and this is probably more likely explained by the fact that for normal functioning of membrane receptors, the presence of VDR is required, as was shown in osteoblasts (Zanello and Norman, 2004). Intraperitonially injection of 1,25$(OH)_2$D (I) resulted in a significant elevation of seizure threshold; however, the increase was transient, lasting only 30 min.

The protective action of 1,25 $(OH)_2$D in the brain, such as reducing the expression of L-type calcium channels in hippocampal neurons (Brewer et al., 2001), plays an important role in supporting neuronal survival. PTZ-induced seizures lead to oxidative brain damage (Ilhan et al., 2005) and 1,25$(OH)_2$D may be effective against the oxidative stress related to these seizures. During seizure activity, calcitriol could control detoxification processes within the brain, through increasing the production of gamma-glutamyl transpepdase (Garcion et al., 1999; Garcion et al., 1996), an enzyme involved in glutathione metabolism. Glutathione is involved in the elimination of reactive oxygen and nitrogen species. Perhaps the higher mortality number in our non-treated animals and VDR mutants occurred due to an uncontrolled formation of reactive oxygen species, known to be associated with hypoxia (Liu et al., 2005).

Another hypothesis could be that 1,25$(OH)_2$D is involved in the regulation of neurotrophic factor synthesis (Garcion et al., 2002). It is known that during seizures, there are changes in the expression of nerve growth factor (NGF), basic fibroblast growth factor (bFGF) and brain derived neurotrophic factor (BDNF), which are thought to contribute to the plasticity of an injured brain (Martin et al., 1995). This suggests that 1,25$(OH)_2$D and VDR may regulate seizure activity through neuroprotective effects (see (Halasz and Rasonyi, 2004; Pitkanen, 2002) for details).

There are well known strain differences in the sensitivity to PTZ (File et al., 1985; Sugaya et al., 1986). In our study, we tested different genetic backgrounds of mice (I, II), concluding that 70 mg/kg of PTZ evoked seizures in 100% of each strain. The anticonvulsive effects of 33 µg of 1,25$(OH)_2$D was effective in both strains (I: NMRI and NMRIx129S1) with administration 40 min (but not 3, 6, 12 or 24 h) prior to seizure induction, thus indicating a high responsiveness of both strains to calcitriol. Overall, the evidence obtained in the present study supports the role of VD and VDR in the regulation of seizure activity in a PTZ mouse model, and suggests the efficacy of VD in the suppression of seizures.

9.3 BALANCE PERFORMANCE IS AFFECTED IN VDR MUTANT MICE (V)

We showed that VDR KO mice have a rather poor balance and motor performance, including poor swimming, impaired motor skills, and disturbed postural control. Several previous studies demonstrated impaired motor coordination and reduced locomotor activity

in different tests (Burne et al., 2006; Burne et al., 2005; Kalueff et al., 2004a), but without a focus on the effects of VD/VDR on balance regulation. Indeed, abnormal vertical swimming and a prevalence of sinking in both VDR KO mouse strains is possibly related to a disturbed vestibular/balance function (Kaiser et al., 2001; Kesby et al., 2006; Lim, 1984; Sawada, Kitahara, and Yazawa, 1994). Moreover, VD also regulates the expression of ECaC1 mRNA in the semicircular canal (Yamauchi et al., 2005), raising the possibility of the involvement of VDR in regulation of vestibular functions (even though the vestibular organ of VDR KO mice was morphologically normally developed, as the stereocilia of hair cells had normal development) (V).

The aim of this study was to further characterize the balance phenotype of VDR KO and 1α-OH KO mice in known tests and also in new tests developed within the study. These tests, such as the tilting box and rotating tube allow assessments of motor coordination, postural and dynamic balance functions. Accelerating rotarod speed is a widely used test to evaluate motor coordination (Crawley, 2000).

Using rotarod test, we demonstrated that 129S1 VDR KO mice have impaired motor coordination, aligning our data with previously reported weak motor abilities of VDR KO mice on a mixed 129Sv and C57BL/6J genetic background (Burne et al., 2005). The tilting box performance showed low postural and balance control of 129S1 VDR KO mice, whilst the NMRI strain demonstrated slightly worse balance performance. Swimming ability was affected in both VDR KO strains. Interestingly, 1α-OH KO mice exhibited normal motor and balance behavior in all tests. Overall, our findings suggest that VDR KO mice have poor swimming abilities. Motor impairment and impaired postural control were present in one of the strains, allowing us to conclude that a lack of VDR could affect motor and balance behavior, and that a balance deficiency could contribute to higher number of falls seen in clinical patients with VD deficit (Montero-Odasso and Duque, 2005; Moylan and Binder, 2007).

In humans, applying VD and calcium supplementation showed a better reduction in the number of falls than calcium alone (Bischoff-Ferrari et al., 2006). Moreover, VD can also decrease falls by itself (Gallagher, 2004). Numerous data show that VD is responsible for muscle strength (Inderjeeth et al., 2007). An *in vitro* study (Endo et al., 2003) showed that VDR KO mice developed skeletal muscle abnormalities independently of secondary metabolic changes (hypocalcaemia and hypophosphatemia), and these changes were accompanied by a deregulated pattern of muscle gene expression. Moreover, they showed that the absence of VDR caused abnormalities in muscle also in older (8 weeks old) rescued VDR KO mice. Treatment with $1,25(OH)_2D$ of VDR-positive myoblastic cells caused a down regulation of several myogenic regulatory factors (Endo et al., 2003). These findings may explain the reduced motor functions and impaired balance in our VDR KO mice. Therefore, the short retention on the rotarod and falling at small angle caused by lower muscle strength in our VDR KO mice.

Swimming inability is a well-known consequence of vestibular defects in rodents (Sawada, Kitahara, and Yazawa, 1994; Sondag et al., 1998; Truett et al., 1994). Mutant mice with otoconial deficiencies have difficulties in keeping a horizontal body position during swimming. Moreover, they have posture, locomotion, balance and spatial orientation problems (Lim, 1984), however our morphological study of the vestibular organ did not demonstrate any morphological abnormalities.

Moreover, VD has an important role in prenatal brain development (Eyles et al., 2003; Eyles et al., 2005; Feron et al., 2005; Garcion et al., 2002). This, as well as previously reported thalamic calcification in VDR KO mice (Kalueff et al., 2006a), suggests that the lack of VDR could cause disturbance in the transmission of vestibular information. Vestibular information is transmitted to cerebellum, cortex, and spinal cord via the thalamus to striatum (Shiroyama et al., 1999). These brain areas are involved in the regulation of balance, movement and walking (Sakka and Vitte, 2004), and disturbances in vestibular transmission in VDR KO mice may be the reason for reduced balance functions. Although reduced balance functions of NMRI VDR KO mice were not statistically significant, they still showed abnormal swimming phenotype, consistent with their impaired balancing.

Notably, Burne *et al* (Burne et al., 2006) showed unaltered swimming abilities in VDR KO mice on a mixed 129S1 x C57BL/6J genetic background. Swimming problems in our VDR KO mice appeared after 5 month of age, whereas Burne et al. used younger mice, which together with a background strain difference could explain the unaltered swimming in these mice. However, we did not find any significant correlation between age and balance performance in other tests. VD related disorders mostly affect elderly people (Gallagher, 2004; Pfeifer, Begerow, and Minne, 2002; Verhaar et al., 2000). Comparing two groups of the same age, only VDR KO mice showed vertical swimming and diving, but not the WT mice.

In conclusion, abnormal motor function, coordination and possible vestibular dysfunction seem to explain the disturbed balance in mice with a VDR mutation, and as a consequence they were then unable to swim and retain posture. This data suggest that VDR and 25(OH)D are crucial for motor coordination and balance.

9.4 THE ROLE OF VDR IN HEARING (III)

Auditory brainstem response (ABR) thresholds were significantly elevated in VDR KO mice tested before 6 months of age, whereas adult WT mice retained normal hearing responses at approximately 30 dB (IV, Fig 2). The importance of VDR for the regulation of normal hearing was supported by our findings of VDR expression in the mouse cochlea (in contrast, cochlear morphology results showed no difference between cochlear structures of WT and VDR KO mice). A study of the expression of genes involved in the regulation of hearing function was also performed, showing that VDR KO mice had normal cochlear expression of the connexin 26 protein, and KCNJ10. Normal expression of connexin 26 indicates that the GJB2 gene was not disrupted VDR KO mice. Levels of potassium were also unaffected by VDR genetic ablation, which is important in the normal development of cochlear hair. Moreover, expression of TRPV4 was not affected, although the activation of caspase 3 in the spiral ganglion of normal and mutant mice was altered.

Interestingly, several previous findings in humans showed an association between VD deficiency and deafness (Brookes and Morrison, 1981; Brookes, 1983; Brookes, 1985). For example, patients with low plasma 25-OH-D show cochlear deafness (Brookes and Morrison, 1981; Brookes, 1983; Brookes, 1985). Likewise, in rats, a VD-deficient diet caused a reduction in Ca^{++} concentration in the perilymph of cochlea (Ikeda et al., 1987). While our VDR KO mice were fed with a rescue diet (to normalize plasma calcium levels), caspase-3 activation was decreased in VDR KO mice cochlea. The caspase-3 KO mice

also develop hearing loss, with a marked impairment of the inner ear and brain morphology (Morishita et al., 2001; Takahashi et al., 2001). Moreover, it was shown that caspase-3 KO mice have insufficient neurotrophic factors in the organ of Corti and spiral ganglion. It was also demonstrated that caspase 3 activation decreased in the cochlea due of aging (Nevado et al., 2006; Riva et al., 2007), what allows us to hypothesize that these changes could also cause the hearing loss in our VDR KO mice. Since deficiency of neutrophin 3 (NT-3) causes degeneration of spiral ganglion (Ernfors et al., 1994; Ernfors et al., 1995; Farinas et al., 1994), and VD is known to regulate the expression of neutrophins, including NT-3 (Brewer et al., 2001; Dusso, Brown, and Slatopolsky, 2005; Eyles et al., 2003; Feron et al., 2005; Garcion et al., 2002; Kiraly et al., 2006; Neveu et al., 1994), these neuroprotective effects of VD may also play a role.

In summary, our findings show that VD/VDR deficiency can cause impairment of hearing in adult mice, including age-related hearing loss already overt at 6 months of age. These data suggest an important role of VDR in the regulation of normal hearing.

9.5 OTHER SENSORY DOMAINS

Since VDR is expressed throughout the olfactory system (Glaser et al., 1999), it was possible to expect that VD and VDR are important for olfactory functions. Although the sensory functions of VDR KO mice were interesting to study, our experiments show that the mouse olfaction and gustation were not affected by VDR genetic ablation (IV). Unaltered olfaction and vision were also reported in these mice previously, using different tests (Kalueff, 2005).

9.6 GENETIC BACKGROUND OF VDR MUTANT MICE

In general, almost all behavioral traits are regulated by environmental and genetic influences. It is clear that behaviors of mice are affected by their environment, as testing members within the same inbred strain does not yield identical scores, despite the fact that their genotype is the same. Studies presented here were performed on mice with genetic mutations of the VDR/VD system, using 3 various background mouse strains. As genetic mutations can alter the nervous system (and thereby affect mouse behavior), the genes from background strains may too interact with the mutated gene, thus further complicating interpretation of the mutant phenotype. The mouse strains, 129S1/SvImJ, NMRI and C57BL/6, which we used as background strains, are widely utilized in behavioral neuroscience. (Burne et al., 2005; Contet, Rawlins, and Deacon, 2001; Cook et al., 2002; Crawley, 2000; Crawley, 1999; Dardenne et al., 2001; Liu and Gershenfeld, 2003; Montkowski et al., 1997; Rodgers et al., 2002; Wahlsten et al., 2003; Yoshizawa et al., 1997). They differ markedly in activity and emotionality: high activity in C57Bl/6, NMRI, BALB; high anxiety in 129S1 and BALB (Crawley, 2000; Kalueff et al., 2007; Kalueff and Tuohimaa, 2005a; Kalueff and Tuohimaa, 2005b).

We used mostly male mice in the present study, although VDR KO female mice did not show any profound differences when compared with males in behavioral tests (Kalueff, 2005). We used mice because mutant mice are much more widely used in neuroscience research than other mammal species, and because they represent excellent translational

models, sharing many brain functions (such as anxiety, hunger, circadian rhythm, aggression, memory, and sexual behavior) with humans (Crawley, 2000; Cryan and Holmes, 2005; Van Meer and Raber, 2005). They are also relatively cheap, easy to house, manipulate and handle.

10 SUMMARY AND CONCLUSIONS

1. Even when VDR KO mice are normacalcemic, they show a variety of subtle behavioral abnormalities, including affected emotional behavior. As shown in this study, VDR genetic ablation causes aberrant neophobic responses to novel food, whilst spatial memory, olfaction, gustation, and depressive-like responses did not appear affected in our mice.

2. Increased seizure sensitivity in VDR KO mice, increased thresholds in mice treated by 1,25$(OH)_2$D, as well higher mortality in VDR mutants and non-treated animals, indicate a possible regulation by the VD/VDR neuroendocrine system of brain seizure activity.

3. VDR KO mice showed an aberrant phenotype in the swimming test, as well as abnormal balance in the accelerating rotarod and tilting box tests. Interestingly, VDR KO mice on two different genetic backgrounds showed similar abnormal swimming. Although morphology of the vestibular organ did not show any difference between genotypes, our data strongly implicate the VDR/VD system in the regulation of vestibular/balancing functions, motor coordination and postural control.

4. Unlike VDR KO mice, 1 α-OH KO mice showed normal swimming, rotarod, tilting box and rotating tube performance, suggesting that the 1,25 $(OH)_2$D metabolite is not crucial for balance functions.

5. As we showed here, VDR is expressed in the cochlea, suggesting that VDR is involved in hearing processes. Indeed, the increased ABR threshold levels in our VDR KO mice, observed before 6 months of age, suggest that our VDR KO mice develop progressive sensorineuronal hearing loss. Although the morphology of cochlea was normal, the activation of caspase-3 was altered in VDR KO mice, and may contribute to their progressive age-related hearing loss.

6. Collectively, our data continue to support the important role of both VD and VDR in the nervous system, including several key domains of behavior, such as emotionality, motorisensory functions. The results of this dissertation will likely have high translational value, as they may explain numerous behavioral and neurological deficits associated in patients with genetically- or environmentally-mediated VD/VDR dysfunctions.

11 ACKNOWLEDGMENTS

This work was carried out in the Department of Anatomy at the Medical School of the University of Tampere. It was financially supported by the Tampere University Hospital Medical Research Fund (EVO), the Academy of Finland, and Tampere Graduate School in Biomedicine and Biotechnology (TGSBB), Tampere, Finland.

I would like to express my graditude to people without whom my thesis would not be possible. The first person to whom I would like to express my deepest appreciation is my supervisor Professor Pentti Tuohimaa. I am indebted to him for his inspiration, constant enthusiasm, patient guidance, helpful comments and criticism, for sharing with me excellent knowledge of the Vitamin D field, and for being such a good example for us all in many aspects of life. It was both a pleasure and honor to work in his group.

I express my deepest appreciation to my co-supervisor Professor Ilmari Pyykkö for introducing me into the field of otovestibular physiology, and for being a nice and supportive mentor. I enjoyed conversations with him while working on our projects, and hope that our collaboration will continue after my graduation.

I am also grateful to another of my co-supervisors, Professor Allan Kalueff (Georgetown University, USA), for his always being so friendly, helpful and reliable. I thank Allan for teaching me plenty of things within behavioral neuroscience, and for showing me a real dedication to work and science. I hope he will always be as full of life as he is now. I am also looking forward to our future collaboration and long-lasting friendship.

I would like to express my gratitude to Professor Seppo Parkkila, for giving me the opportunity to conduct my thesis in the department of Anatomy, Medical School, University of Tampere. My sincere gratitude goes to Professors Simo Oja and Pirjo Saransaari, for their support at early beginning of my PhD studies. I also thank the members of my doctoral dissertation committee, PhD Heimo Syvälä and Professor Markku Pelto-Huikko, for their advice and comments during my project. I was fortunate to have a very supportive and experienced committee, willing to contribute their time and knowledge to ensure my success. I thank Heimo very much, for his valuable help with morphological analyses. I am also grateful to Professor Howard T. Jacobs for his excellent lectures and courses in the field of Immunology, Genetics and Molecular Biology.

I express my gratitude to the reviewers of this thesis PhD Sami Väisänen and MD, PhD Vootele Voikar for their critical reading of the thesis and useful comments.

My sincere thanks to my colleague, co-author and friend PhD Yan-Ru Low, for many helpful scientific discussions and support. I would also like to thank my other co-authors PhDs Jing Zou, Esko Toppila, MSc Jing-Huan Wang, and Ya Zhang. Special thanks to my collaborator, co-author and friend, Ms. Tiina Keisala, for her cooperation, friendship and support in many aspects. I appreciate her talking to me everyday in Finnish (Lämpimän kiitokset kaikesta!), and am looking forward to our future collaboration and friendship.
I would like to thank Ms. Arja Ahola (for her helping me whenever I asked for it, and for nice chats we had together) and Ms. Marianne Kuuslahti (for her technical assistance). My warmest appreciation to all other colleagues and friends in the Medical School of the University of Tampere: Pasi Pennanen, Tommi Manninen, Sami Purmonen, Riina

Sarkanen, Ulla Järvelin, Hilkka Mäkinen, Mirja Hyppönen, Nadezda Nazarova, Andrei Taranukhin, and Anastasia Galeeva. I wish to thank Marina Toompuu from IMT for her friendship, and am grateful to MSc Eloice Kok and BA Peter Hart, for revising the language of this thesis.

My thanks to a dear friend Natalia Zinevych for her support, kindness, as well as for her good mood and always having a nice word to say. It is so hard to be far away from you! I would like to thank my friends from North Ossetia: MDs Dudajti Laura and Anatoli Kusrati, for their great professional advice in psychiatric aspects, which I used in analyzing mouse behavior. I would also like to thank my Armenian friends Professor Karen Egiazarian and MD Alina Grigorian, for their taking care, and celebrating events together. My sincere thanks to the Leon Orbeli Institute of Physiology of the Armenian National Academy of Sciences, and to all my teachers and colleagues in Yerevan, Armenia.

I am thankful to my mother-in-law, Gohar, my sister-in-law, Hasmik, her husband, Eduard, and their charming daughter Emmy for their support, understanding and being kind to me. My special and warm thanks to my uncle Samvel and aunt Janna for their unlimited love, devotion and faith in me. I wish to express my thank to Samvel for introducing me to science, and for great enthusiasm he passed on to me.

My thanks, with love, to my parents - Silva and Georgi - for everything they did for me. My dear mother, you are a beautiful and intelligent lady and you always gave the best example to me. I am also grateful to my lovely sisters, Susanna and Lilit for their love, help and support. I wish them luck in their lives and scientific careers.

Finally, my loving thanks to my husband David and my daughter Goharik for their love, patient understanding and their belief in me. You make me so happy every day, and are the true meaning of my life!

Tampere, Finland
03.12.2008

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Brain Research Bulletin 67 (2005) 156–160

BRAIN RESEARCH BULLETIN

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Anticonvulsant effects of 1,25-dihydroxyvitamin D in chemically induced seizures in mice

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Received 7 April 2005; received in revised form 12 June 2005; accepted 13 June 2005
Available online 26 July 2005

Abstract

Here, we study the role of a neurosteroid hormone Vitamin D in epilepsy. To examine this problem, we used 1,25-dihydroxyvitamin D, an active form of Vitamin D, injected subcutaneously to NMRI mice (33 μg/20 μl) 40 min prior to seizures induced by systemic injection of pentylenenetrazole (PTZ, 70 mg/kg). Overall, compared to the vehicle-treated control animals ($n = 11$ in each group), the Vitamin D-treated mice demonstrated reduced severity of PTZ-induced seizures (longer latency, shorter duration and lower mortality). In a separate experiment, we assessed the time-course of antiepileptic effects of 1,25-dihydroxyvitamin D. For this, we injected this compound (33 μg/20 μl) to NMRIx129S1 mice ($n = 11$) 40 min, 3, 6, 12 and 24 h prior to seizures, showing that antiepileptic effects were short-term, almost disappearing 3 h after administration. Our findings show that Vitamin D plays a direct anticonvulsant role in the brain and suggest that the Vitamin D endocrine system may represent a new target for the development of anticonvulsant drugs.

Keywords: Vitamin D; 1,25-Dihydroxyvitamin D; Epilepsy; Chemically induced seizures; Mice

1. Introduction

Numerous data indicate that the brain represents a target tissue for Vitamin D actions [4,8,21]. This steroid hormone plays an important role in the nervous system including differentiation, regulation of Ca^{2+} homeostasis, modulation of neurotrophins release and activity of key brain genes and enzymes of neurotransmitter metabolism [4,8,31]. The functions of Vitamin D are mediated through the nuclear Vitamin D receptor (VDR), a member of the nuclear receptors superfamily of ligand-activated transcription factors [3,15]. VDR are widespread in the brain and the spinal cord, implying the potential role of both Vitamin D and VDR in the brain [4,8]. 1,25-Dihydroxyvitamin D (1,25-D, calcitriol) is the main biologically active metabolite of Vitamin D and the principal ligand for VDR, exerting its effects in a manner similar to other steroid hormones [3,8,22].

There are several lines of evidence that link various Vitamin D-related disorders to epilepsy. Low Vitamin D leads to hypocalcemia able to induce seizures due to hyperexcitability of the neural membranes [4,9]. In humans, seizures accompanied by hypocalcemia and lowered Vitamin D levels are often seen in patients with hereditary or nutritional rickets [10–14,20,24–28]. In line with this, Vitamin D and calcium therapy have long been known to reduce seizures by relieving hypocalcemia in such patients [2,9,10]. Taken together, this allowed to discuss the possibility of anticonvulsant action of Vitamin D [5], possibly due to its positive effects on mineral and hormonal homeostasis. At the same time, it has long been known that chronic treatment with antiepileptic drugs impairs mineral homeostasis in epileptic patients, leading to a marked hypocalcemia and reduced plasma levels of Vitamin D (which in turn may increase seizure) [1,6,7,12,20,23–27]. These observations have led to a wide practice of using Vitamin D as an additional therapy in epilepsy [1,6,14,23,26].

However, there is mounting evidence indicating that Vitamin D per se may play a significant role in the physiological

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0361-9230/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.brainresbull.2005.06.022

mechanisms underlying various brain disorders. For example, Vitamin D dysfunctions have recently been suggested to play a role in pathogenesis of multiple sclerosis, schizophrenia, anxiety and depression, rev.: [4,8,15,17]. As a physiologically active neurosteroid hormone, Vitamin D is also involved in multiple neuroprotective mechanisms (rev. in [8,15,31]). Can the Vitamin D system be involved in epilepsy? In addition to clinical findings described earlier, there are some animal data that directly support this notion [29,31]. In their pioneering study, Siegel et al. [29] reported direct anticonvulsant effects (increased seizure threshold in rats following the electrical stimulation of the dorsal hippocampus) within 5–10 min after i.c.v. or i.v. injection of 1,25-D. Thus, the potential role of Vitamin D in epilepsy, and the possibility of direct anticonvulsant properties of this neurosteroid hormone, seem to justify further experimental investigation.

In the present study, we examined the effects of 1,25-D administration on chemically induced seizures in mice. Since pentylenetetrazole (PTZ), a potent blocker of the chloride ionophore at the gamma-aminobutyric acid GABA-A receptors, is the most frequently used chemoconvulsant in experimental models of epilepsy [16,19], we used this model in our experiments. Given a relatively high toxicity of 1,25-D due to its well-known hypercalcemic effects, we used 33 μg/20 μl of the drug in the present study, basing our choice on the earlier data [29] using systemic doses and injection into the brain of 50–100 μg of this drug in rats. Choosing the pre-treatment time for our studies, we considered earlier findings that 1,25-D may exert its anticonvulsant effects within 30–180 min in the electroconvulsive model of seizures in rats [29]. Here, we demonstrate that 1,25-D leads to reduced severity of seizures and lower mortality in the model of PTZ-induced seizures in mice.

2. Materials and methods

Subjects were 22 adult NMRI and 87 adult NMRIx129S1 mice (30–35 g; University of Tampere, Finland) maintained in a standard virus/parasite-free facility (temperature: 22 ± 2 °C; humidity: 55 ± 5%) and exposed to a 12-h light:12-h dark cycle. Lights were turned off at 18.00 and on at 06.00 h. The animals were experimentally naïve and housed in groups of five, with food and water freely available. The testing was always conducted between 14:00 and 18:00 h. On the day of the experiments, animals were transported to the experimental room and left undisturbed for 1 h for acclimation. In Experiment 1, we tested NMRI mice, a strain widely used in epilepsy research, including PTZ-induced seizures (e.g., [30,32]). 1,25-D (33 μg/20 μl (Leo Pharma, Denmark); 4 nM solution in 20% isopropanol) was injected to 11 NMRI mice s.c. using a 50 μl Hamilton syringe. Control mice ($n = 11$) received the same volume of isopropanol. After injection, the animals were returned to their cages for recovery. Forty minutes later each animal received a bolus of i.p. injection of PTZ (Sigma, UK; 70 mg/kg) and was placed into a clean glass cylinder (diameter: 20 cm; height: 30 cm) for observation of seizure profile. Between subjects, the cylinder was thoroughly cleaned with wet/dry cloths and 70% ethanol, to remove any olfactory cues.

Seizures and seizure latency times were observed visually over a 15-min observation period and analysed by a trained observer (blind to the treatment groups) sitting in front of, and 1 m away from, the testing cylinder. The latencies (s) of the first twitch, Straub tail, oro-facial, clonic and tonic seizures were analysed in both groups of mice, and reckoned as 900 s (total observation time) in the mice not showing the respective behaviours. Mortality in both groups was assessed over a 30-min period. An animal was considered dead if the heart was not beating upon manual check (the latency of death was reckoned as 1800 s if the animals remained alive after a 30-min observation period). The intensity of seizures was registered using a modified Racine's scoring system [16]: 0 (no response), 1 (freezing), 2 (head nodding or isolated twitches), 3 (oro-facial seizure), 4 (clonic seizure), 5 (tonic seizure), 6 (death). Clonic seizures consisted of rhythmic contractions of forelimb and/or hind-limbs. Tonic seizures consisted of rigid extension of the fore- and/or hind-limbs with or without posture loss.

In a separate experiment (Experiment 2), we wanted to assess the time-course of antiepileptic effects of 1,25-D, also testing its properties in a different mouse strain. For this, we used hybrid NMRIx129S1 mice injecting them with 1,25-D (33 μg/20 μl) or vehicle 40 min, 3, 6, 12 and 24 h prior to PTZ-induced seizures ($n = 11$ in each group), and recording their seizure profiles as described earlier. The latencies (s) of the first twitch, oro-facial, clonic and tonic seizures were analysed in both groups of mice. Mortality in both groups was also assessed over a 30-min period.

To assess the role of Ca^{2+} (Experiment 3), 21 NMRIx129S1 mice were injected with the same dose of 1,25-D (40 min and 3 h prior to sampling) or vehicle ($n = 7$ in each group). Animals were then sacrificed and their blood samples taken to measure plasma Ca^{2+} levels (mmol/l) using atomic absorption spectroscopy (Yhtyneet Laboratoriot, Helsinki, Finland).

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. All results are expressed as mean ± S.E.M. Data were analysed by the one-way ANOVA test (Experiment 2, factor: treatment) and the Mann–Whitney *U*-test for independent samples (Experiments 1–3). A probability of less than 0.05 was considered statistically significant.

3. Results

The results of Experiment 1 are summarized in Table 1. Overall, 1,25-D produced a dramatic decrease in seizure

Table 1
Reduced susceptibility to pentylenetetrazole (70 mg/kg i.p.)-induced seizures in NMRI mice treated with 1,25-dihydroxyvitamin D (33 μg/20 μl s.c.) 40 min prior to testing

Measures	Control mice ($n = 11$)	Drug-treated mice ($n = 11$)
Latency to the first twitch (LT) (s)	56 ± 14	77 ± 25
Latency to the Straub tail (s)	51 ± 8	96 ± 24*
Latency to oro-facial seizure (LOF) (s)	62 ± 9	93 ± 15*
Latency to clonic seizure (LC) (s)	89 ± 20	130 ± 24
Latency to tonic seizure (LT) (s)	169 ± 29	181 ± 33
Latency to death (LD) (s)	839 ± 198	1743 ± 38***
Number of mice with twitches	10/11	9/11
Number of mice with Straub tail	10/11	9/11
Number of mice with oro-facial seizures	10/11	9/11
Number of mice with clonic seizures	10/11	8/11
Number of mice with tonic seizures	9/11	5/11
Mortality rate (MR)	6/11	2/11
Duration of oro-facial seizures (s)	8 ± 2	8 ± 2
Duration of clonic–tonic seizures (s)	32 ± 4	10 ± 3***
Total duration of seizures (s)	40 ± 5	18 ± 3**
Average Racine's score	5.7 ± 0.21	4.4 ± 0.40*

Data are the means ± S.E.M. Fractions represent the number of mice showing different stages of seizures (of the total number of mice in the group).
* $P < 0.05$ difference between the groups (*U*-test).
** $P < 0.01$ difference between the groups (*U*-test).
*** $P < 0.005$ difference between the groups (*U*-test).

severity in PTZ-treated mice. While the duration of relatively mild oro-facial seizures was similar in both groups, the duration of more severe clonic–tonic and the total duration of seizures were markedly shorter in the mice treated with 1,25-D. As can be seen in Table 1, there was a clear tendency to longer latencies to the first twitch, Straib tail ($P < 0.05$), oro-facial ($P < 0.05$, *U*-test), clonic and tonic seizures in this group. Mortality rate was trice higher in the control group (55% versus 18%) and there was a significantly shorter latency to death compared to the 1,25-D-treated group ($P < 0.005$, *U*-test; Table 1). Finally, the average Racine's score was significantly lower in the 1,25-D-treated mice. Taken together, these data clearly indicate a robust and fast anticonvulsant action of 1,25-D in mice.

The results of Experiment 2 are summarized in Table 2. Overall, 1,25-D produced clear anticonvulsant effects in NMRIx129S1 mice if injected 40 min but not 6, 12 or 24 h prior to PTZ administration (see Table 2 for the results of data analysis using one-way ANOVA). 1,25-D (s.c. 40 min prior to PTZ) significantly delayed the onset of clonic, tonic seizures and death (Table 2). These results confirm data obtained in Experiment 1 in NMRI mice, showing similar effects of acute 1,25-D treatment in both strains. In contrast, a 3 h pre-treatment with 1,25-D produced only mild non-significant reduction of seizures in mice, compared to their vehicle-treated controls.

Finally, plasma Ca^{2+} levels were unaltered in the 3 h pre-treatment group (2.34 ± 0.06 versus 2.47 ± 0.14 mmol/l in controls, NS), showing only mild but non-significant 30% increase in the 40 min pre-treatment group (2.75 ± 0.24 mmol/l; Experiment 3).

4. Discussion

In general, our findings demonstrate acute anticonvulsant effects of 1,25-D in the model of chemically-induced seizures in mice, and are in line with previously published studies in rats in the model of hippocampal seizures [29]. In this early study, stereotaxic injection into hippocampus or i.v. injection of 50–100 μg of 1,25-D (but not Vitamin D or 25-hydroxyvitamin D) elevated seizure thresholds in rats following electrical stimulation of dorsal hippocampus—the effect lasting for 30 min (i.v.) or 180 min (i.c.v.). Explaining this and our findings, we first noted that the ability of 1,25-D to reduce seizures in the present study occurred within a relatively short (40 min) time following s.c. administration of the drug. This effect is in line with fast non-genomic action of this hormone, suggesting that its slower genomic effects may not be involved in its antiepileptic profile reported here (Table 1) and in the previously published studies [29]. Our present data extend the generality of these observations, allowing us to speculate that "fast" anticonvulsant properties of 1,25-D may represent a general pharmacological profile of this drug in different rodent models of epilepsy.

Overall, several potential mechanisms may underlie the antiepileptic activity of 1,25-D. For example, it is possible to assume that this steroid hormone may modulate the

Table 2
Susceptibility to pentylenetetrazole (70 mg/kg i.p.)-induced seizures in NMRIx129S1 mice treated with 1,25-dihydroxyvitamin D (33 μg/20 μl s.c.) 40 min, 3, 6, 12 and 24 h prior to testing ($n = 11$ in each group)

Mea sure	Control mice	Pre-treatment groups					
		40 min	3 h	6 h	12 h	24 h	*F*
LT	52 ± 13	96 ± 24	60 ± 15	50 ± 14	86 ± 31	58 ± 10	i
LOF	58 ± 17	106 ± 27	71 ± 16	72 ± 27	97 ± 30	73 ± 15	ii
LC	64 ± 17	165 ± 34*	78 ± 21	78 ± 16	130 ± 21	92 ± 18	iii
LT	220 ± 35	427 ± 91*	250 ± 40	209 ± 17	231 ± 46	201 ± 22	iv
LD	718 ± 159	1187 ± 153*	800 ± 162	638 ± 92	741 ± 207	515 ± 83	v
MR	10/11	9/11	11/11	10/11	9/11	11/11	

Measures as in Table 1. *F*—the results of one-way ANOVA test (factor: treatment): (i) $F_{(5,66)} = 0.99$, $P = 0.429$ (NS); (ii) $F_{(5,66)} = 0.63$, $P = 0.68$ (NS); (iii) $F(5,66) = 3.07$, $P = 0.016$ (<0.05); (iv) $F(5,66) = 3.12$, $P = 0.014$ (<0.05); (v) $F(5,66) = 2.33$, $P = 0.053$ (clear tendency).
* $P < 0.05$ difference from the control group (*U*-test).

brain neuromediators and receptors (see [4,8] for details). Since GABA-A receptors represent an important target for non-genomic action of many neurosteroids and neuroactive hormones [18], 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 [8]. Given the crucial role of GABAergic system in epilepsy pathogenesis, and the specific GABA-inhibiting action of PTZ, the possibility of steroid-like "fast" effects of 1,25-D on GABA-A receptors may need further experimental investigation in detail. Since 1,25-D represents a neuroactive/neurosteroid hormone (i.e. acting and synthesised in the brain [4,15,21]), this possibility seems indeed likely.

Since numerous clinical data show anticonvulsant effects after Vitamin D therapy [1,5,6,26], it was also possible to assume that 1,25-D may affect seizures by acting via VDR to induce certain brain genes including those encoding key cytokines and enzymes of neurotransmitter metabolism [4,8]. Interestingly, for example, 1,25-D is known to down-regulate interleukin 6, recently reported to be involved in epilepsy and exert pro-convulsant effects in PTZ-induced seizures in rats [15,17]. However, the lack of anticonvulsant properties following 3–24 h pre-treatment with 1,25-D clearly negates the role of "slow" mechanisms, such as genomic effects, in this action of Vitamin D.

Since Vitamin D plays an important role in the regulation of Ca^{2+} homeostasis, another possibility for its anticonvulsant action can be altered Ca^{2+} metabolism. For example, 1,25-D has rapid effects on Ca^{2+} absorption from the intestine and other organs [12]. Therefore, it is possible to assume that 1,25-D may lead to increased plasma and reduced brain Ca^{2+} concentrations, thus contributing to overall reduction of neuronal excitation during PTZ-induced seizures. To test this hypothesis, we measured plasma Ca^{2+} levels in vehicle versus 1,25-treated mice (40 min and 3 h pre-treatment time), showing only mild non-significant transient 30% increase in plasma Ca^{2+} following 40 min, but not 3 h pre-treatment. This suggests that calcemic effects of this hormone may be dissociated from its antiepileptic action. In line with this notion, it was shown previously that Vitamin D treatment with 4000–16,000 IU/day led to robust clinical antiepileptic effects not related to altered plasma Ca^{2+} levels (see [1] for review). Finally, Vitamin D has also long been known to indirectly reduce the levels of Ca^{2+} in the brain (rev. in [8,15]) by stimulating the expression of several Ca-binding proteins and inhibiting the expression of L-type Ca^{2+} channels. However, our data clearly negate this hypothesis since a longer pre-treatment time with 1,25-D (needed for such effects on expression) did not reduce seizures (Table 2).

Importantly, the calcium level is not the only determining factor for the occurrence of seizures in clinic [1]. Indeed, while some seizures initially do not respond to Ca^{2+} therapy but are easily corrected with Vitamin D, individual thresholds may also be an important factor for seizure pathogenesis in humans [1] or animals [29,33]. Our present study, analysing a wide range of seizure parameters over a long period of time (Tables 1 and 2) revealed altered thresholds in mice, as assessed by their seizure latencies. It is, therefore, possible to suggest that Vitamin D, perhaps acting in a neurosteroid-like manner, may be involved in "fine tuning" of neuronal excitability, thus regulating epileptogenesis at the threshold level. In line with this hypothesis, lower circulating Vitamin D levels are reported to increase antiepileptic efficiency of 1,25-D [29]. Collectively, these data suggest possible modulation of seizure activity by Vitamin D in mice.

Comparing the data obtained in Experiments 1 and 2 (Tables 1 and 2), we note general similarity in antiepileptic effects of 33 μg 1,25-D 40 min prior to PTZ, as assessed by delayed latencies (% of the respective vehicle-treated controls, 100%): twitch (138% and 184%, NS), oro-facial (150% $P < 0.05$ and 183%, NS), clonic (146%, NS and 258%, $P < 0.05$), tonic (107%, NS and 194%, $P < 0.05$) seizures and death (208%, $P < 0.005$ and 165%, $P < 0.05$). These observations further confirm robust antiepileptic effects of Vitamin D. However, the two mouse strains used here also demonstrated some differences in their seizure profiles. For example, both NMRIx129S1 mouse groups exhibited a stronger epileptic phenotype (mortality rate 9–10/11, Experiment 2) than did the NMRI groups (2–6/11, Experiment 1), also showing more individual variability (as assessed by SEM scores) in their seizure responses. These data suggest that genetic background of mice (influencing their sensitivity to PTZ, also see [33]), may also affect their responsivity to antiepileptic action of 1,25-D.

From this point of view, it may be interesting to compare several different popular mouse strains (e.g., 129S1, C57Bl6, Balb/c [33]) in their sensitivity to antiepileptic action of 1,25-D. Moreover, it may also be important to examine the role of VDR in epilepsy by analysing susceptibility to various seizures in mutant mice lacking VDR receptor gene—the animal model of Vitamin D-related rickets. These mice are currently available for biomedical research and have already been reported to show several brain dysfunctions [17]. Given the well-known link between rickets and seizures [4,10,14], testing these mice in various models of epilepsy may represent an important area of research. These studies, already underway in our laboratory, will allow to further assess the exact mechanisms of Vitamin D antiepileptic action, dissecting genomic (VDR-dependent) versus non-genomic effects of 1,25-D.

In addition, it may also be feasible to assess seizures in mice or rats chronically deprived of Vitamin D—another useful animal model of Vitamin D-related dysfunctions. Using a combination of Vitamin D deprivation with or without calcium deprivation, such studies may allow to assess the role of calcium in acute antiepileptic actions of Vitamin D. Furthermore, testing potential antiepileptic properties of other endogenous Vitamin D-related compounds (e.g., 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D) may also represent an important area of epilepsy research. Collectively, these findings may enable the search for novel antiepileptic drugs based on selective non-toxic synthetic

Vitamin D-related ligands (e.g., [31]). For example, finding a steroid Vitamin D-related compound with both Vitamin D-like and GABA-modulating properties, if successful, could lead to a highly effective therapy targeting several parallel pathogenic mechanisms of epilepsy.

In summary, here we have demonstrated a clear anticonvulsant action of 1,25-D in the model of PTZ-induced seizures in mice, consistent with the previously published clinical and pre-clinical data [5,29]. In our study, 1,25-D affected predominantly the more severe stages of seizures (Tables 1 and 2), showing the anticonvulsant profile which is clinically relevant and may be of interest for potential application. Taken together, these findings provide a neurobiological rationale for the use of Vitamin D in epilepsy—not only as a supplementary calcium-normalizing agent, but as an active antiepileptic compound per se. Overall, this study further outlines the important role of Vitamin D in the regulation of seizures, supporting the notion that the Vitamin D endocrine system may play a significant role in the physiological mechanisms underlying epilepsy [29,31].

Acknowledgements

This study was supported by the University of Tampere, the Medical Research Fund of Tampere University Hospital (EVO) and the Academy of Finland.

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Increased severity of chemically induced seizures in mice with partially deleted Vitamin D receptor gene

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Received 31 July 2005; received in revised form 14 September 2005; accepted 3 October 2005

Abstract

Vitamin D is a neuroactive steroid hormone with multiple functions in the brain. Numerous clinical and experimental data link various Vitamin D-related dysfunctions to epilepsy. Here, we study the role of Vitamin D receptors (VDRs) in experimental epilepsy in mice. To examine this problem, we assessed the seizure profiles in VDR knockout mice following a systemic injection of pentylenetetrazole (70 mg/kg). Overall, compared to the wild-type (WT) 129S1 mice ($n = 10$ in each group), the VDR knockout group significantly demonstrated shorter latencies to the onset, higher Racine scores and increased mortality rates. Our findings suggest that VDRs modulate seizure susceptibility in mice, and that the Vitamin D/VDR endocrine system may be involved in the pathogenesis of epilepsy.

Keywords: Vitamin D; Vitamin D receptors; Epilepsy; Knockout mice; Chemically induced seizures

Vitamin D is a steroid hormone with multiple functions in the nervous system including the regulation of differentiation, Ca^{2+} homeostasis, neurotrophins release and activity of the key brain genes [4,7,12,14,21]. The functions of Vitamin D are mediated through the Vitamin D receptor (VDR), a member of the nuclear receptors superfamily of ligand-activated transcription factors [9,26,29,30]. VDR are widespread in the brain and spinal cord, implying that they have a role in the regulation of brain functions [7,14,21].

A growing body of literature suggests a link between Vitamin D-related disorders and epilepsy [1,2,7,25]. Seizures due to low Vitamin D, common in patients with hereditary or nutritional rickets [1,2,16,19,20,40,41], are reduced by Vitamin D, underlining the possibility of the anticonvulsant properties of this hormone [8,10,39]. Moreover, direct anticonvulsant effects of 1,25-dihydroxyvitamin D, an active hormonal form of Vitamin D, have been reported in rats and mice [25,42] in various experimental models of epilepsy, further confirming the role of the Vitamin D/VDR system in epilepsy.

Genetically targeted animals provide a powerful tool to study the neural mechanisms of epilepsy [36,47]. Mice with genetically impaired VDR (knockout mice, KO) are currently available for biomedical research focusing on the biological functions of Vitamin D and VDR. Several groups have generated four VDR KO mice strains (Tokyo, Munich, Boston and Leuven mice) by the targeted disruption of different fragments of the VDR gene [11,26,27,30,45,48]. VDR KO mice generated in Tokyo [48] express truncated VDR (with intact ligand-binding domains but ablated DNA-binding domains), unable to activate gene expression [5]. Since the absence of functional VDR results in target-tissue insensitivity to Vitamin D, testing these VDR KO mutant mice in different models of epilepsy may be an important tool to assess the role of the Vitamin D/VDR system in epileptogenesis.

The unique physiology of these mice (lacking functional VDR and characterized by elevated plasma Vitamin D [26,30,48]) allows us to dissect different mechanisms of Vitamin D action in epilepsy. Given its anticonvulsant properties [8,42], reduced seizure activity in these mutants would indicate VDR-independent anti-epileptic action of Vitamin D. In contrast, higher seizure activity in these mice would support the role of VDR and VDR-mediated mechanisms in epilepsy.

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0304-3940/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2005.10.007

Here, we tested the Tokyo VDR KO mice in the model of chemically induced seizures. Pentyleneterazole (PTZ), a potent blocker of the chloride ionophore at the gamma-aminobutyric acid (GABA-A) receptors, was chosen for its common use in epilepsy research [22,35]. Our study shows that genetic disruption of the VDR gene increases the severity of PTZ-induced seizures and mortality in mice.

Subjects were adult female mice (20–25 g; 3–3.5 months old; University of Tampere, Finland) maintained in a standard virus/parasite-free facility (temperature, 22 ± 2 °C; humidity, 55 ± 5%) and exposed to a 12-h light and 12-h dark cycle. Lights were turned off at 18:00 and turned on at 06:00 h. VDR KO mice were bred from the line initially generated in the University of Tokyo [48], and compared to their WT littermates of 129S1 mouse strain ($n = 10$ in each group). Mice of both the genotypes were produced by four to five heterozygous crosses. Tail clips were taken for genotyping performed using the polymerase chain reaction (PCR) on DNA prepared from tail tissue. Four primers were used to amplify a 130 bp VDR band and a 450 bp Neo band from the targeted gene [6]. On day 21, postpartum pups were weaned and assigned to different cages based on their genotype and gender. The animals were experimentally naïve and housed individually, with food and water freely available. To normalize mineral homeostasis in the VDR KO mice, they were fed a special rescue diet (2% Ca, 1.25% P and 20% lactose supplemented with 2.2 IU Vitamin D/g; Lactamin AB, Sweden) [31]. Since a Ca-rich diet may lead to several physiological alterations in the WT but not the VDR KO animals (e.g., [3]), which may confound our results, an additional control group (WT fed with the rescue diet) was not used in the present study.

In all these mice, the occurrence of spontaneous seizures was assessed daily during the homecage observations (30 min/animal/day) for 5 days prior to the testing. The testing was conducted between 14:00 and 18:00 h. On the day of the experiments, the animals were transported to the experimental room and left undisturbed for 1 h for acclimation. During this period, the occurrence of spontaneous seizures was also monitored in all these mice. One hour later, each animal received a bolus of i.p. injection of PTZ (Sigma, UK; 70 mg/kg), and was placed in a clean glass cylinder (diameter, 20 cm; height, 30 cm) for observation of the seizure profile. The convulsant dose of PTZ (70 mg/kg) was chosen for our experiments, based on its use in epilepsy research in mice [35] and its ability to induce pronounced seizures in 129S1 mice (own systematic observations). Between the subjects, the cylinder was thoroughly cleaned with wet/dry cloths and 70% ethanol to remove any olfactory cues. Seizures and seizure latency times were observed visually over a 30-min observation period and analysed by a trained observer (intra-rater reliability >0.9) sitting in front of, and 1 m away from, the testing cylinder. The latencies of the first twitch, orofacial, clonic and tonic seizures were analysed in both the groups of mice, and reckoned as 1800 s (total observation time) in the mice not showing the respective behaviours. The intensity of the seizures was registered using a modified Racine's scoring system [22]: 0, no response; 1, freezing; 2, head nodding or isolated twitches; 3, orofacial seizure; 4, clonic seizure; 5, tonic seizure; 6, death. Clonic seizures consisted of rhythmic contractions of forelimbs and/or hindlimbs. Tonic seizures consisted of rigid extension of the forelimbs and/or hindlimbs with or without posture loss. Mortality in both the groups was also assessed over a 30-min period. An animal was considered dead if the heart was not beating upon manual checkup (the latency of death was reckoned as 1800 s if the animals remained alive after a 30-min observation period). In addition, blood samples were taken after PTZ injection in nine KO and seven WT mice, to measure the baseline plasma Ca^{2+} levels by atomic absorption spectroscopy (Yhtyneet Laboratoriot, Helsinki, Finland). Blood was taken immediately for those mice of both genotypes, which died from the seizures within 10 ± 5 min (Racine score, 6); others were duly sacrificed and the same procedure was performed.

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. All results are expressed as mean ± S.E.M. Data were analysed using the Mann–Whitney *U*-test for independent samples. Correlation between the plasma Ca^{2+} levels and seizure measures was analysed using the Spearman rank-order correlation coefficient (R). In all the tests, a probability of less than 0.05 was considered statistically significant.

The results of this study are summarized in Table 1. While the durations of orofacial, clonic and tonic seizures (as well as total duration of the seizures) were similar in both the groups, the latency measures were significantly shorter in the KO group for twitches and tonic ($P < 0.05$, *U*-test) but not for orofacial seizures ($P > 0.05$, *U*-test), suggesting that VDR mutation may affect seizures in this study at the threshold level. In line with this, there was a clear tendency to shorter latencies to the clonic seizures ($P = 0.08$, *U*-test) and higher mortality rate ($P = 0.06$,

Table 1
Increased susceptibility to pentylenetetrazole (70 mg/kg i.p.)-induced seizures in the Vitamin D receptor knockout (VDR KO) female mice, compared to their wild-type (WT) littermates

Measures	WT ($n = 10$)	VDR KO ($n = 10$)
Latency to the first twitch (s)	66.9 ± 4.5	50.4 ± 4.4*
Latency to orofacial seizure (s)	73.8 ± 5.3	63.2 ± 4.9
Latency to clonic seizure (s)	521 ± 220	179 ± 74&
Latency to tonic seizure (s)	701 ± 246	201 ± 80*
Latency to death (s)	1259 ± 226	429 ± 167*
Number of mice with twitches	10/10	10/10
Number of mice with orofacial seizures	10/10	10/10
Number of mice with clonic seizures	8/10	10/10
Number of mice with tonic seizures	7/10	10/10
Mortality rate	4/10	9/10&
Duration of orofacial seizures (s)	9 ± 1.4	8.7 ± 1.6
Duration of clonic seizures (s)	10 ± 3	13 ± 3
Duration of tonic seizures (s)	12 ± 4.6	14 ± 3
Total duration of seizures (s)	31 ± 5.8	35.7 ± 5.5
Average Racine's score	4.9 ± 0.4	5.9 ± 0.10*

* $P < 0.05$; difference between the groups (*U*-test). Data are the means ± S.E.M. Fractions represent the number of mice showing different stages of seizures (of the total number of mice in the group).
& Robust trend ($P = 0.05$–0.08, *U*-test).

U-test) in these mutants. The average Racine's score was significantly higher in the mutant group ($P < 0.05$, *U*-test), clearly indicating increased sensitivity to PTZ seizures in the VDR KO mice. There was also a significantly shorter latency to death compared to the WT controls ($P < 0.05$, *U*-test; Table 1), further confirming more severe seizures in the VDR KO group.

No spontaneous seizures were observed in any of these mice in the present study (data not shown). Plasma Ca^{2+} levels, effectively normalized by rescue diet, were only slightly lower (2.22 ± 0.10 mmol/l KO, 2.64 ± 0.09 mmol/l WT, $P > 0.05$, *U*-test) in the VDR KO group, and similar to the normal levels (2.35–2.37 mmol/l) typical for the background 129S1 strain [36]. In addition, Spearman correlation analysis showed no significant correlation between the plasma Ca^{2+} levels and total duration of seizures ($R = 0.14$ and -0.43 for WT and KO, respectively; $P > 0.05$), average Racine scores ($R = 0.67$ and 0.65 for WT and KO, respectively; $P > 0.05$) and mortality rate ($R = 0.67$ and 0.05 for WT and KO, respectively; $P > 0.05$).

Overall, the present study is the first report analysing the seizure sensitivity in VDR KO mice. Interestingly, these mice did not show spontaneously occurring seizures here, nor during the extensive testing in a battery of behavioural tests in our previous studies [23,24]. However, our present results using the chemically induced seizures directly link genetic ablation of VDR to increased seizure susceptibility in mice, and confirm the role of VDR in the brain mechanisms underlying epilepsy. These findings are consistent with the previously published studies, showing the anticonvulsant effects of Vitamin D in different rodent models of the seizures [25,42], and in patients with hypovitaminosis D and rickets [8,10].

In general, several potential physiological mechanisms may explain our findings. For example, since PTZ acts via the GABA-A receptors [22,35,43], it was possible to link higher seizures in VDR KO mice to the altered GABAergic system. Indeed, the Vitamin D status has been recently reported to be positively correlated with the expression of α4, and slightly correlate with the expression of α1, subunits of GABA-A receptors [12]. Thus, the lack of Vitamin D/VDR signalling in our VDR KO mice may disrupt such upregulation, leading to the altered expression of these subunits in the brain. Given the crucial role of the GABAergic system in epilepsy pathogenesis and the key role of alpha subunits in GABA-A receptor functioning, modulating their sensitivity to GABA-lytic convulsants, such as PTZ [43], this possibility seems indeed likely.

It was also possible to assume that VDR genetic ablation may affect seizures by disrupting VDR-mediated modulation of certain brain genes [7,12,14]. For example, Vitamin D downregulates proconvulsant cytokine IL-6, and upregulates anticonvulsant growth factors GDNF (glial cell derived neurotrophic factor) and NT3 (neurotrophin-3) [14,21,22,34,46]. Likewise, Vitamin D stimulates the expression of Ca-binding proteins, such as parvalbumin and calbindins [14,21], also known to exert anti-epileptic effects [28]. Thus, genetic disruption of the VDR gene in mice may affect Vitamin D-modulated expression of these molecules. Indeed, a reduction in the expression of brain calbindin D9k has already been shown in VDR KO mice [31]. Thus, it is tempting to speculate that altered baseline levels of endogenous convulsants and anticonvulsants in these mice may contribute to the overall increase in the seizure susceptibility observed here (Table 1). In addition, VDRs are involved in the multiple mechanisms of neuroprotection, see refs. [7,14,21]. Since the link between the neuroprotective and anti-epileptic mechanisms has long been recognized [15], the reduction of VDR-mediated neuroprotective tone in VDR KO may also contribute to their increased seizure susceptibility.

The existence of non-nuclear (membrane) VDR (VDRm) has been widely debated [14,38], but still remains obscure [9]. If the anti-epileptic action of Vitamin D [21,42] is mediated via VDRm independently of VDR, mice with high Vitamin D levels, no VDR and intact VDRm, such as VDR KO, might be expected to display reduced seizures phenotype. Our results reporting increased seizure profiles in VDR KO mice, negate this possibility, suggesting that VDR-independent mechanisms are not involved in the altered seizure phenotype reported here. Interestingly, recent data suggest that VDR and VDRm may represent the same receptor protein, and show that VDR are required for both genomic and non-genomic effects of Vitamin D [18,37,49]. Our results do not contradict these findings, and it is therefore possible to assume that both genomic and non-genomic VDR-dependent mechanisms (affected in the VDR KO mice) may contribute to the phenomenon reported here.

Furthermore, since both Vitamin D and VDR play an important role in the regulation of Ca^{2+} homeostasis [7,9,33], another possibility for the increased seizure susceptibility in our VDR KO mice can be hypocalcemia [3,6,48], contributing to an overall increase in neuronal excitation. To minimize this factor, we used a special diet, normalizing Ca^{2+} metabolism in VDR KO mice [31]. In the present study, both the groups were essentially normocalcemic and showed plasma Ca^{2+} levels close to the normal levels reported for the WT 129S1 strain [36]. Furthermore, there was no correlation between the plasma Ca^{2+} and seizure intensity in any of the two groups, indicating that hypocalcemia may not be involved in the phenomenon reported here. Likewise, although not directly tested here, it is possible to assume that VDR KO mice may have several additional physiological anomalies (such as altered biochemistry and metabolism) which could alter drug pharmacokinetics, thus contributing to the genotype difference observed here in response to PTZ. Clearly, this possibility requires further indepth investigation in these mutant mice.

Moreover, analysing our data, we note that the genes of background strains may influence mouse sensitivity to PTZ [13,36]; thus, interacting with the mutation effects. The WT 129S1 strain is known to be relatively sensitive to seizures [13,36], and was therefore appropriate for the present study. However, it may also be interesting to assess seizures in VDR KO on a mixed (e.g., [6]), or other isogenic (e.g., [27]) genetic backgrounds, especially those which differ markedly in their seizure profiles [13,36,44]. We are currently transferring the VDR null mutation to several new genetic backgrounds (C57Bl/6, Balb/c and NMRI), in order to perform such comparative studies. Moreover, it may also be important to examine the role of VDR in epilepsy by analysing the seizure susceptibility in mutant mice with other abnormalities in the Vitamin D system. For example,

mice lacking 1α-hydroxylase, a key enzyme of Vitamin D bioactivation [9], are currently available for biomedical research [26]. Displaying the phenotypes resembling the clinical abnormalities observed in the patients with rickets [26], these mice may be a useful tool to further dissect the role of the Vitamin D system in epilepsy. Moreover, it may also be feasible to assess the seizures in mice or rats chronically deprived of Vitamin D—another useful animal model of Vitamin D-related dysfunctions [12].

Finally, we would underline that our findings are limited to only one (PTZ) model of seizures. It is widely accepted that PTZ-induced seizures are an experimental model of human myoclonic and absence epilepsy [17,32]. Therefore, testing other convulsant drugs (e.g., pilocarpine or kainate) in our VDR KO mice, as well as using other experimental models of epilepsy, would help to delineate the generalizability of the potential role of Vitamin D and VDR in various types of epilepsy disorder.

In conclusion, we show that VDR KO mice display increased susceptibility and higher mortality in the model of PTZ-induced seizures (Table 1). These data are consistent with the previously published clinical and preclinical data [7,8,25,42] linking Vitamin D to epilepsy, and may be associated with the disturbed VDR-mediated signalling pathways. Overall, this study further supports the notion that the Vitamin D/VDR endocrine system may play a significant role in the physiological mechanisms underlying epilepsy [42].

Acknowledgements

This study was supported by the University of Tampere, the Medical Research Fund of Tampere University Hospital (EVO) and the Academy of Finland. We are greatly indebted to Professor Shigeaki Kato (University of Tokyo, Japan) for providing the initial VDR mutant mice for our research.

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Original Paper

Audiology & Neurotology

Audiol Neurotol 2008;13:219–230
DOI: 10.1159/000115431

Received: July 13, 2007
Accepted after revision: October 19, 2007
Published online: February 7, 2008

Progressive Hearing Loss in Mice with a Mutated Vitamin D Receptor Gene

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Key Words
Calcium · Ganglion cell · Hearing loss · Vitamin D · Caspase

Abstract
Background: Both hypo- and hypervitaminosis D can cause sensorineural hearing loss, and aural symptoms due to vitamin D insufficiency are especially common during gravidity. Hormonal forms of vitamin D regulate transcription by binding with the high-affinity vitamin D receptor (VDR). ***Objective:*** To assess the effects of impaired vitamin D action in VDR knockout (KO) mice on hearing, cochlear morphology, and cochlear gene expression. ***Materials and Methods:*** Eighteen young male and female mice (10 VDR KO and 8 wild type, WT, ≤6 months old), 33 adult male and female mice (16 VDR KO and 17 WT, between 7 and 14 months old), and 11 aged male and female mice (5 VDR KO and 6 WT, ≥15 months old) on 129S1 genetic background were studied. Auditory thresholds were evaluated by auditory brain stem response. Morphological changes were analyzed using plastic embedding and light microscopy. The expression of key genes (known to play a role in the regulation of cochlear function), and caspase 3 activity, were assessed using immunofluorescent confocal microscopy. ***Results:*** There was a statistically significant difference between the young and the adult groups, and between the adult and aged groups of WT mice. There was also a statistically significant difference between the adult and aged groups in VDR KO mice, and between the young WT group and the young VDR KO group. Spiral ganglion cell loss was observed in the basal turn of adult VDR KO mice, a phenomenon infrequently found in WT mice. Expression of connexin 26, KCNJ10, and transient receptor potential channel vanilloid subfamily 4/6 was not affected by VDR KO-mediated hearing loss. Caspase 3 activation was detected in the spiral ganglion cell and its satellite cells, stria vascularis, spiral ligament fibrocytes, and the organ of Corti in both genotypes. However, the percentage of positive cells and the staining intensity were lower in the VDR KO (compared to the WT) mice. ***Conclusion:*** These data suggest that sensorineural hearing loss progressively developed at an earlier age in VDR KO mice. While the fundamental gene expressions in the cochlea were not influenced by VDR mutation, it resulted in decrease of caspase 3 activation, which may be one of the factors underlying accelerating age-related hearing loss observed in VDR KO mice.

Introduction

The active forms of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25(OH)2D_3] and 25-hydroxyvitamin D_3, have several important biological roles including the regulation of calcium homeostasis, cellular differentiation, in-

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hibition of tumor cell proliferation, immune function, and brain development [McGrath et al., 2004; Veenstra et al., 1998]. All these actions are at least partially mediated by activation of the vitamin D receptor (VDR), a member of the steroid/nuclear receptor superfamily of ligand-activated transcription factors [Dusso et al., 2005; Holick, 2004, 2006; Lou et al., 2004; Ylikomi et al., 2002]. Vitamin D deficiency is a common factor in the reduction of bone-mineral density, and is associated with an increased risk of several chronic age-related diseases [Javaid et al., 2006; Zittermann, 2003]. Vitamin D deficiency, VDR malfunction, hypoparathyroidism, and hypervitaminosis have long been suggested to be potential causes of sensorineural hearing loss [Brookes and Morrison, 1981; Brookes, 1983, 1985; Cohen et al., 1979; Ikeda et al., 1989, 1987; Ishida et al., 2001; Ziporyn, 1983].

Mutant mouse lacking functional VDR – VDR knockout (KO) mouse – is a powerful tool for exploring the vital functions of vitamin D. Nevertheless, VDR KO mice require a special diet for survival, a problem that has only recently been solved [Li et al., 1998]. The behavioral phenotype of VDR KO mice include muscular and motor impairments, swimming disturbances and increased anxiety, reported by our group [Kalueff et al., 2004, 2006b] and further studied by others [Burne et al., 2006, 2005].

Our previous study also showed that a severe calcification developed in VDR KO mice in the thalamus [Kalueff et al., 2006a] – the brain structure that projects to the inferior colliculus of the auditory pathway and connects with the auditory cortex, as has recently been extensively investigated in both animals and humans [de la Mothe et al., 2006; Devlin et al., 2006; Pearson et al., 2007; Sigalovsky and Melcher, 2006; Takayanagi and Ojima, 2006]. Upon sustaining damage, neuronal retrograde degeneration was observed in the auditory system, and auditory brain stem response (ABR) amplitude decreased after inferior colliculus ablation [Kaga et al., 1999; Yamada et al., 2000]. Vitamin D also plays a trophic role in differentiation and maturation of neurons by promoting neurite outgrowth [Brown et al., 2003; Taniura et al., 2006]. Collectively, this suggests that hearing physiology may be affected in individuals with vitamin D/VDR dysfunctions.

In the present study, we analyzed hearing threshold levels and cochlear morphology to evaluate possible changes in the inner ear in VDR KO mice. In addition, gene expression and caspase 3 activation were also studied here.

Materials and Methods

Animals

Eighteen young male and female mice (10 VDR KO and 8 wild type, WT, $\leq$6 months old), 33 adult male and female mice (16 VDR KO and 17 WT, between 7 and 14 months old), and 11 aged male and female mice (5 VDR KO and 6 WT, $\geq$15 months old) were studied. VDR KO mice were initially generated at the University of Tokyo (Japan) [Yoshizawa et al., 1997]. All mice used were littermates on 129S1 genetic background produced by heterozygous crosses.

The VDR KO and WT mice used in the present study were maintained in a virus/parasite-free facility (temperature 24 $\pm$ 1°C, humidity 50 $\pm$ 5%), and exposed to a 12-hour light, 12-hour dark cycle. Lights were turned off at 19:00 and on at 7:00. The animals were experimentally naive and housed individually in transparent plastic cages (13 $\times$ 12 $\times$ 14 cm), with food and water freely available. To eliminate hypocalcaemia and rickets in the VDR KO mice, all mutant animals were fed a special rescue diet containing 2% calcium, 1.25% phosphorus, and 20% lactose (Lactamin AB, Sweden). The plasma Ca^{2+} level was only slightly lower in the VDR KO group (2.11 + 0.26 mM) than in the WT group (2.49 + 0.07 mM), and was also slightly below the normal levels reported previously for adult 129S1 mice (2.31–2.36 mM). All animal experiments were approved by the Ethical Committee of the University of Tampere. Animal care and experimental procedures were conducted in accordance with the European legislation.

Genotyping

On D21 postpartum, pups were weaned and tail clips were taken for genotyping performed using the polymerase chain reaction method on DNA prepared from tail tissue. Four primers were used to amplify a 166-bp VDR band (forward, 5′-CTG CTC TTC TTA CAG GGA TGG-3′, and reverse, 5′-GAC TCA CCT GAA GAA ACC CTT G-3′) and a 400-bp Neo band (forward, 5′-ATC TTC TGT CAT CTC ACC TTG C-3′, and reverse, 5′-CAA GCT CTT CAG CAA TAT CAC G-3′) from the targeted allele. After being genotyped, mice were assigned to different cages based on their genotype.

ABR Measurement

To assess auditory thresholds, BioSig32 (Tucker Davis Technologies, USA) was used for ABR threshold recording in both VDR KO and WT mice under general anesthesia with Domitor (0.8 mg/kg medetomidine hydrochloride) and Ketalar (80 mg/kg ketemine hydrochloride). A click duration of 50 μs and a repetition rate of 21.1/s were used for stimulation. Responses from 512 sweeps were averaged with a gain of 20 at each intensity level using a filter of 0.1–3 kHz. Thresholds were judged by visible repeatable responses.

Plasma Ca^{2+} Level Measurement

Before cardioperfusion, blood was taken from the heart to measure plasma Ca^{2+} level with atomic absorption spectroscopy (Yhtyneet Laboratoriot, Helsinki, Finland).

Plastic Embedding

The animals were perfused with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M PBS (pH 7.4) following cardiac perfusion removal of the blood with 0.01 M PBS (pH 7.4) under general an-

esthesia with pentobarbital sodium (100 mg/kg). The bulla was removed and further fixed with the same fixative solution overnight. After washing with 0.1 M PBS, decalcification with 10% EDTA was performed at room temperature for 3 weeks. Dehydration was carried out by using 35, 70, 90, and 95% ethanol for 10 min each, and absolute ethanol twice for 15 min. After infiltrating with JB-4, samples were embedded at 4°C for 2 days. 2-μm sections were made with a microtone section machine (LEICA RM2265, Germany). The slides were stained with toluidine blue for light microscopy.

Immunofluorescent Confocal Microscopy

The animals were perfused with 4% formaldehyde in 0.1 M PBS (pH 7.4) following cardiac perfusion removal of the blood with 0.01 M PBS (pH 7.4). The bulla was removed and further fixed with the same fixative solution overnight. After washing with 0.1 M PBS, decalcification with 10% EDTA was performed at room temperature for 3 weeks. A standard paraffin embedding procedure was used and the samples were sectioned at 4-μm thickness. For immunofluorescent staining, the sections were heated at 60°C for 2 h, deparaffinized with xylene, and passed through gradient ethanol until a final PBS wash. The sections were digested with 0.1% trypsin at 37°C for 30 min, washed with PBS-T (0.1% Tween 20 was included) for 3 × 2 min, and incubated with 1:20 preimmunized goat serum at room temperature for 30 min. Different primary antibodies were used with incubation overnight at 4°C: rabbit polyclonal antibody to VDR, 1:10 (Abcam, UK, ab12129); mouse monoclonal antibody to synaptophysin, 1:200 (Sigma-Aldrich, USA); rabbit polyclonal antibody to connexin 26, 1:15 (Zymed, USA); rabbit polyclonal antibody to Kir4.1 (KCNJ10), 1:100 (Alomone Labs, Israel); rabbit polyclonal antibody to transient receptor potential channel vanilloid subfamily (TRPV) 4/6, 1:100 (Alomone Labs), and rabbit polyclonal antibody to caspase 3 active, 1:800 (R&D system, Inc., USA). For negative control, the primary antibodies were omitted in VDR, synaptophysin, connexin 26, KCNJ10, TRPV6, and caspase 3 active. The primary antibody was saturated with 0.4 mg/ml TRPV4 peptide containing 1% preinoculated goat serum overnight in the negative control for TRPV4 staining. After washing with PBS-T, the slides were incubated with FITC-conjugated goat antiserum against rabbit IgG (1:80, Sigma-Aldrich), Alexa Fluor® 568-labeled goat antiserum against rabbit IgG (1:400, Invitrogen, USA) or TRITC-conjugated goat antiserum against mouse IgG (1:200, Sigma-Aldrich, USA), which depended on the host primary antibody, at room temperature for 60 min, followed by incubation with 4′,6-diamidino-2-phenylindole (DAPI; 10 ng/ml, Sigma-Aldrich, USA) for 10 min. The slides were mounted with Gel Mount™ Aqueous Mounting Medium (Sigma-Aldrich, USA) after washing with PBS-T.

Confocal Microscopy

The immunofluorescently stained slides were observed under an Olympus microscope IX70 installed with ANDOR IQ (FITC fluorescence at 488 nm with a laser beam; Alexa Fluor® 568 and TRITC fluorescence at 568 nm with a laser beam; DAPI with a 340- to 380-nm filter). For caspase 3 activation quantification, the original confocal microscopy tiff images were evaluated with ImageJ 1.36b software. The positive percentage was calculated on the basis of counting the caspase 3 activation and total nuclear DAPI staining.

Statistics

Binomial test was used to compare the caspase 3 activation percentage between different groups. Student's t test was applied to compare the average ABR thresholds, spiral ganglion cell counting, and caspase 3 activation signal intensity between different groups. All data were presented as means ± SE.

Results

Hearing Loss in VDR KO Mice

An average threshold of 22 ± 1, 29 ± 2 and 73 ± 3 dB SPL was found in the young, adult and aged WT groups, respectively. There was a statistically significant difference between the young and adult WT groups ($p < 0.05$), and between the adult and aged WT groups ($p < 0.01$; fig. 1). In contrast, an average threshold of 32 ± 3, 35 ± 3, and 56 ± 8 dB SPL was found in the young, adult and aged VDR KO groups, respectively. Although there was no statistically significant difference between the young and the adult VDR KO mouse groups, we found a statistically significant difference between the adult group and aged group in VDR KO mice ($p < 0.05$; fig. 1). In addition, there was also a statistically significant difference between the young WT and VDR KO groups ($p < 0.01$), but not between the adult WT versus VDR KO, and aged WT versus VDR KO groups (fig. 1). ABR waveforms in VDR KO mice with hearing loss were disfigured when compared with WT mice (fig. 2a, b).

Cochlear Morphology

The cochlear structures including the organ of Corti, lateral wall, and spiral ganglion cells were fully developed in both WT (fig. 3a) and VDR KO mice (fig. 3b–d). The efferent nerve endings were detected using an antibody against synaptophysin underneath the inner hair cells and the outer hair cells (fig. 3c). An excessive layer of cells was found in the row of the outer hair cells in both VDR KO (fig. 3e) and WT (fig. 3f) mice. This does not seem to be an artifact because the 2-μm thickness of the sections cannot cover the overlap of hair cells. Condensed nuclear staining with toluidine blue of spiral ganglion cells in both VDR KO (fig. 3g) and WT mice (fig. 3h) is a sign of degeneration [Kawamura et al., 1997]. The volume of these cells was also extremely reduced. Numerous spiral ganglion cell losses were seen in the basal turn of adult VDR KO mice, which was infrequently found in WT mice (fig. 3i). The spiral ganglion cells were counted and the VDR-KO mice had, on average, 9.8 cells/mm^2 (SD 3.6) and the WT mice had 15.7 cells/mm^2 (SD 2.6). No statistically significant differences were found because of small sample size.

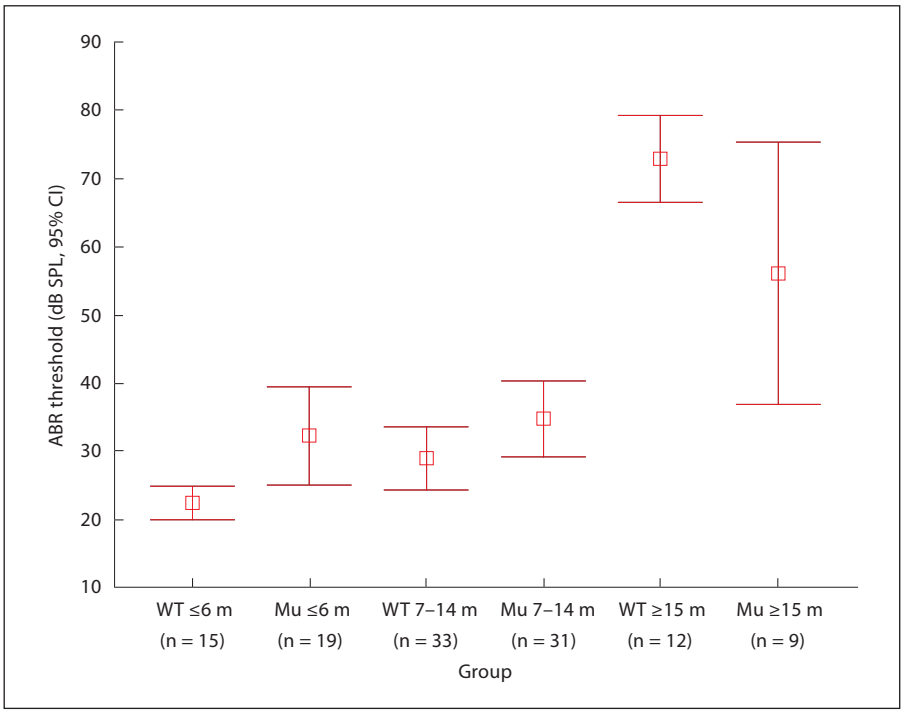

Fig. 1. VDR KO accelerated the development of hearing loss shown by ABR. Age-related ABR threshold elevation was found in both the WT and the VDR KO groups. Hearing loss developed significantly faster in the VDR KO group than in the WT group ≤6 months. Mu = Mutant, VDR KO. The number of ears that were measured is shown in parentheses.

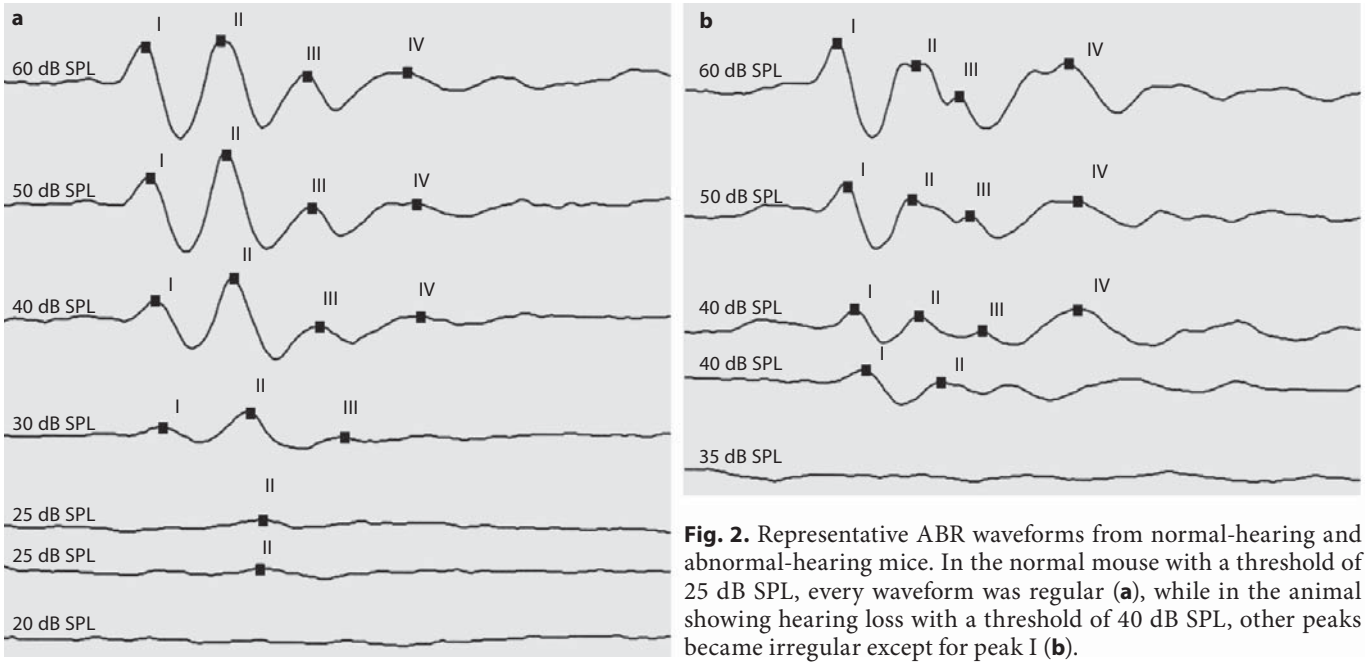

Fig. 2. Representative ABR waveforms from normal-hearing and abnormal-hearing mice. In the normal mouse with a threshold of 25 dB SPL, every waveform was regular (**a**), while in the animal showing hearing loss with a threshold of 40 dB SPL, other peaks became irregular except for peak I (**b**).

VDR Expression in the Cochlea

VDR was expressed in the nuclei of osteocytes of bulla, spiral ganglion cells, and Reissner's membrane cells, in the cytoplasm of spiral ligament fibrocytes and stria vascularis cells, and in both nuclei of the hair cells and inner sulcus cells in WT mice (fig. 4). VDR was not detectable in VDR KO mouse cochlea. There was no signal for VDR in the negative control slice when the primary antibody was omitted.

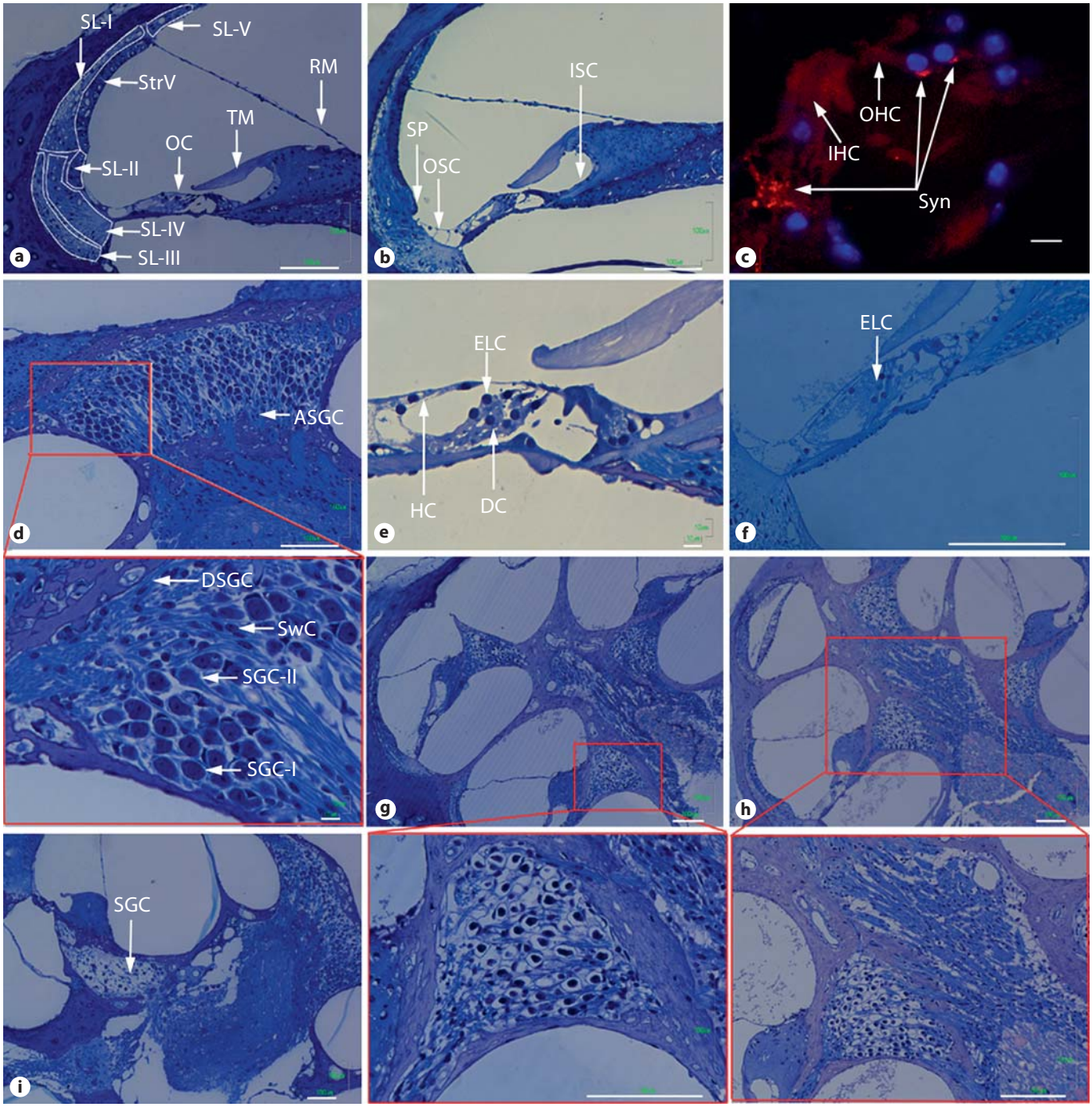

Fig. 3. Comparison of cochlear morphology between VDR KO and WT 129S1 mice. The cochlear structures including the organ of Corti, lateral wall, and spiral ganglion cells are fully developed in both WT mice (**a**) and VDR KO mice (**b–d**). **c** The efferent nerve endings are proven by detecting synaptophysin underneath the inner hair cells and outer hair cells. An excessive layer of cells appears in the row of the outer hair cells in both VDR KO mice (**e**) and WT mice (**f**). Degenerating spiral ganglion cells, which show condensed nuclear staining with toluidine blue and extremely reduced cellular volume, occurred in both VDR KO mice (**g**) and WT mice (**h**). Spiral ganglion cell loss was found in the basal turn of VDR KO mice (**i**). ASGC = Axonal process of the spiral ganglion cells; DC = Dieters cells; DSGC = dendritic process of spiral ganglion cells; ELC = excessive layer of cells; HC = Hensen's cells; IHC = inner hair cells; ISC = inner sulcus cells; OC = organ of Corti; OHC = outer hair cells; OSC = outer sulcus cells; RM = Reissner's membrane; SGC-I = spiral ganglion cell type I; SGC-II = spiral ganglion cell type II; SL-I, SL-II, etc. = spiral ligament fibrocyte type I, type II, etc.; SP = spiral prominence; StrV = stria vascular; SwC = Schwann cells; Syn = synaptophysin. Scale bar = 100 μm (**a**, **b**, **d**, **f–i**), 6.9 μm (**c**), 10 μm (**e**).

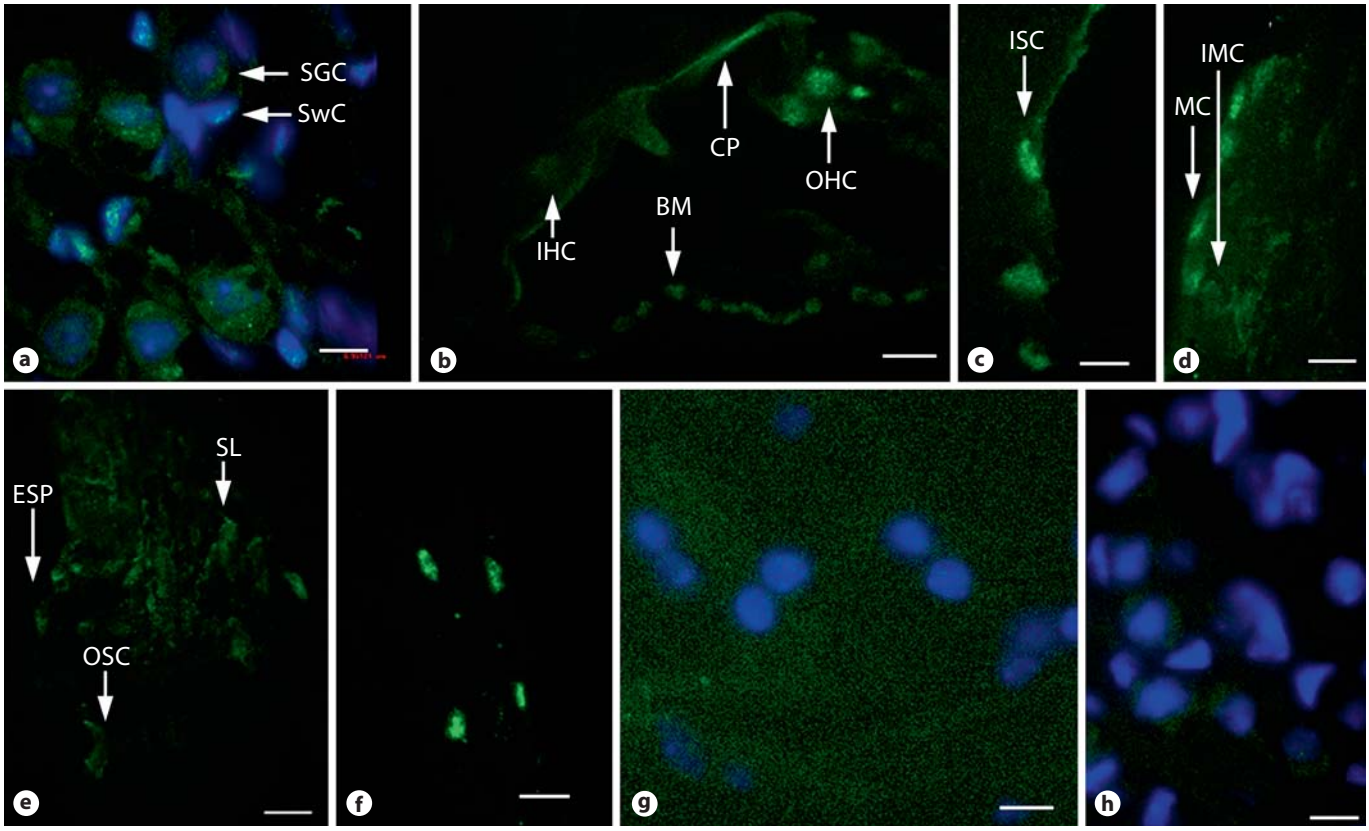

Fig. 4. VDR was expressed in the WT mouse cochlea. **a** VDR protein staining (green) was found in the nuclei of both spiral ganglion cells and Schwann cells. **b** In the organ of Corti, VDR was expressed in the nuclei of hair cells, the basilar membrane, and in the cuticular plate. VDR was also found in the nuclei of inner sulcus cells (**c**), marginal cells and intermediate cells of the stria vascularis (**d**), epithelium of spiral prominence, spiral ligament, and outer sulcus cells (**e**). **f** VDR was also expressed in the nuclei of osteocytes of bulla. **g** VDR was undetectable in VDR KO mouse cochlea. **h** There was no staining when primary antibody was omitted. **a**, **g**, **h** The nuclei were counterstained with DAPI. BM = Basilar membrane; CP = Cuticular plate; ESP = epithelium of spiral prominence; IMC = intermediate cells of the stria vascularis; MC = marginal cells of the stria vascularis; SGC = spiral ganglion cells; SL = spiral ligament. Scale bar = 6.9 μm.

Normal Expression of Connexin 26 and KCNJ10 in the Cochlea

Connexin 26 was weakly expressed in the Deiters' cells and Hensen's cells. Intense staining was found in the Claudius cells, inner sulcus cells, outer sulcus cells, type I spiral ligament fibrocytes, and the capillary of the stria vascularis, in both VDR KO and WT mice similarly (fig. 5a, b). There was no signal for connexin 26 in the negative control slice without the primary antibody. In conclusion, GJB2 gene expression was normal in VDR KO mice.

KCNJ10 expression was detected in the hair cells, supporting cells, inner sulcus cells, intermediate cells of the stria vascularis, and spiral ganglion cells in both WT and VDR KO mice with immunofluorescent confocal microscopy (fig. 5c, d). No KCNJ10 signal was detected in the negative control slice with primary antibody omitted.

Nonaffected TRPV4/6 Expression in the Cochlea

In the WT mouse cochlea, TRPV4 protein appeared in the cuticular plate of both inner and outer hair cells receiving the mechanic stimulation that comes from the hair bundle. Strong expression was also detected in the apical cytoplasm of both inner and outer pillar cells and their junction, inner sulcus cells, interdental cells, and in the matrix of the limbus, Reissner's membrane, spiral prominence, strial vascularis, spiral ligament fibrocytes, and spiral ganglion cells. TRPV4 expression was not changed in the cochlea of VDR KO mice (fig. 5e, f). No signal was found on the slice when the primary antibody

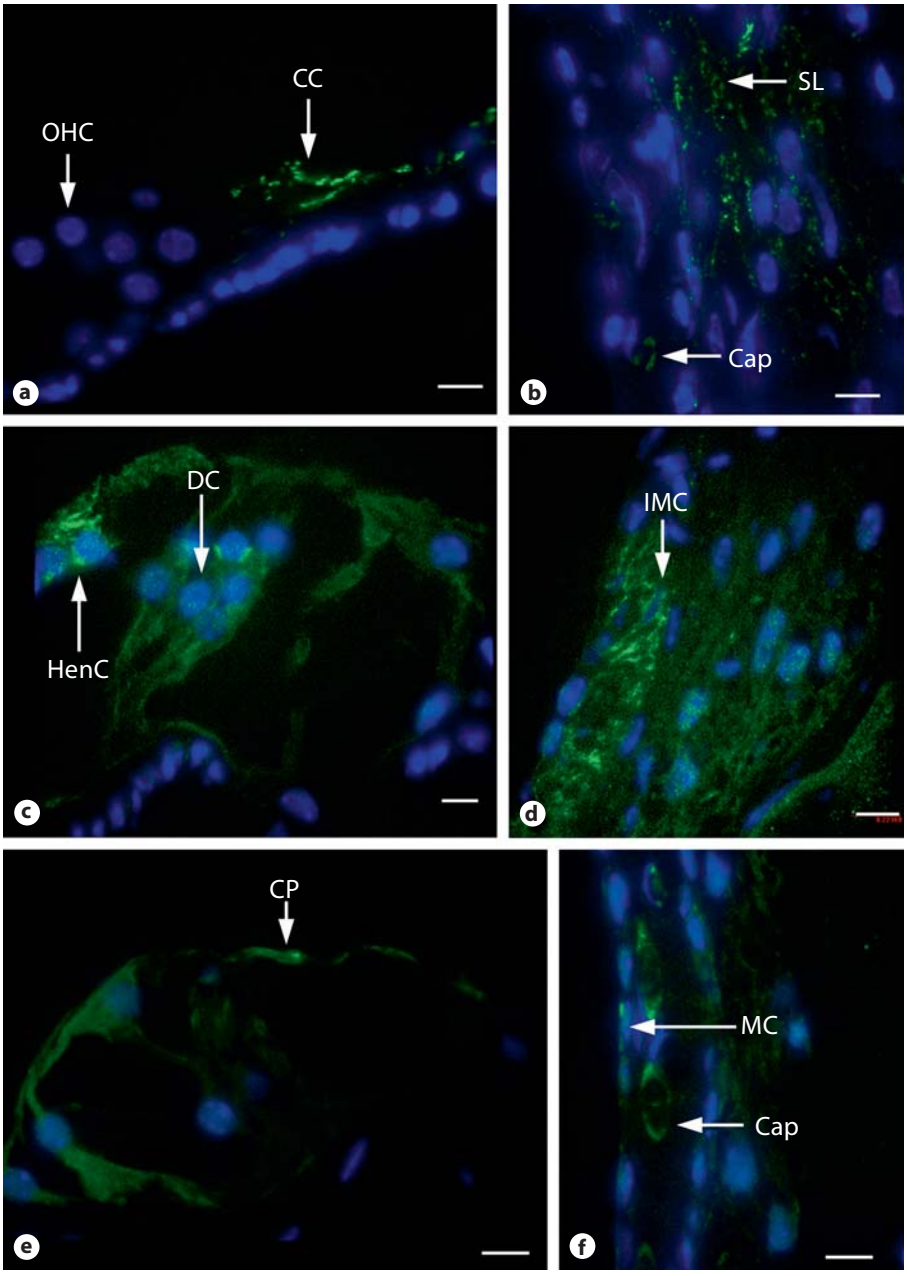

Fig. 5. The cochlear expression of connexin 26, KCNJ10, and TRPV4 was not affected by VDR KO. Connexin 26 protein was detected in Claudius cells in the organ of Corti (**a**), spiral ligament and capillary of the stria vascularis (**b**). KCNJ10 protein was expressed in Hensen's cells and Dieters cells in the organ of Corti (**c**), and marginal cells of the stria vascularis (**d**). TRPV4 was found in the cuticular plate in the organ of Corti (**e**), marginal cells and capillary in the stria vascularis (**f**). Cap = Capillary; CC = Claudius cells; HenC = Hensen's cells. Scale bar = 6.9 μm.

was saturated with TRPV4 peptide. There was very faint and variable expression of TRPV6 in the mouse cochlea.

Caspase 3 Is Activated in the Cochlea of Both WT and VDR KO Mice

Caspase 3 activation was performed in the cochlea of 6.5- and 10-month-old mice in both the WT and VDR KO groups. The activation was detected in cochlea cells, including the spiral ganglion cell and its satellite cells, stria vascularis, spiral ligament fibrocytes, and the organ of Corti (fig. 6). However, as can be seen in table 1, the percentage of positive cells and the staining intensity were lower in VDR KO (compared to the WT) mice. The activated caspase 3 showed a cytosolic and nuclear localization (fig. 6).

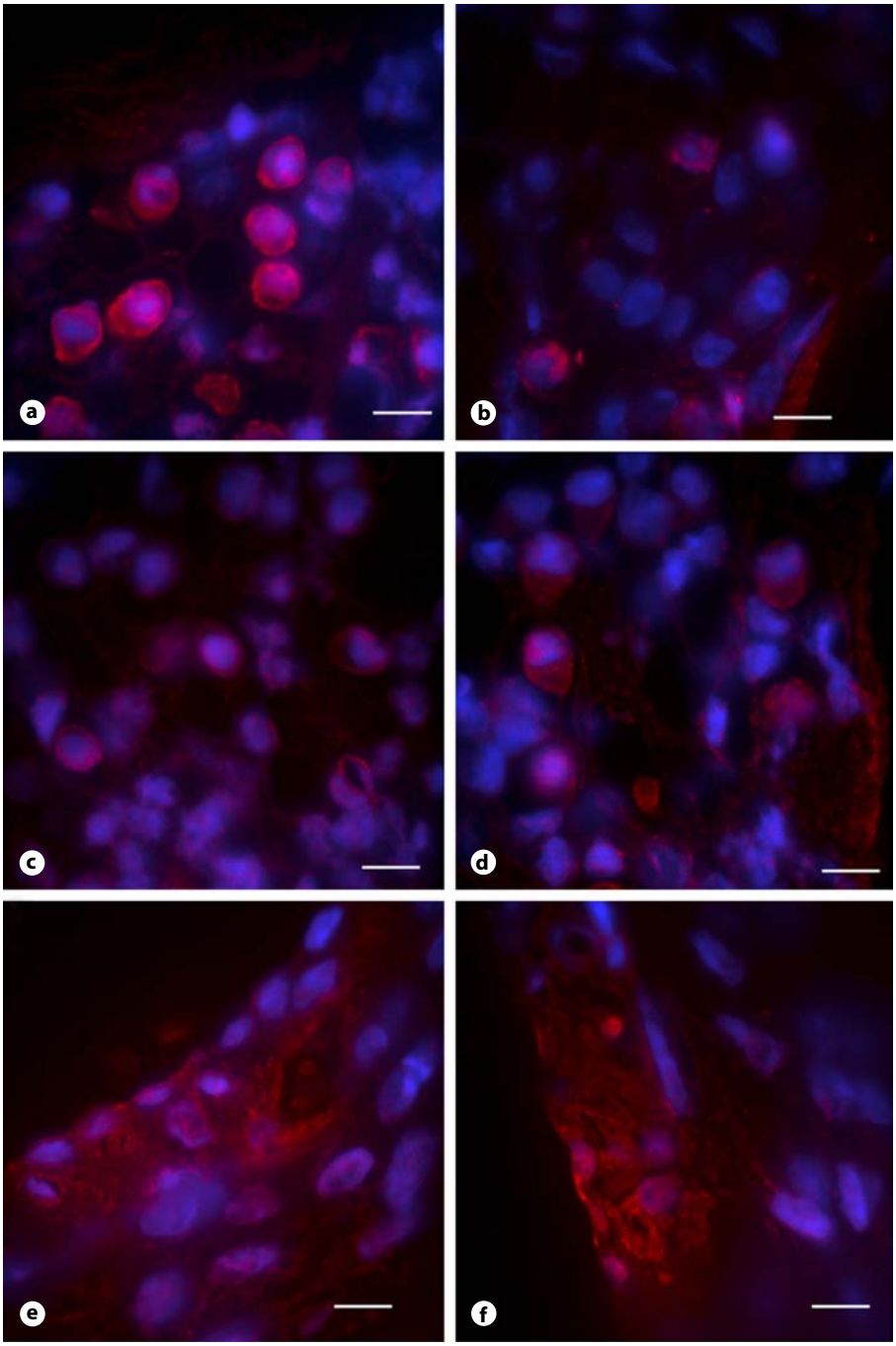

Fig. 6. Caspase 3 activation was decreased in the cochlea of VDR KO, compared to the WT mice. Caspase 3 activation (red) detected in both the cytosol and nucleus (blue) of neuron and satellite cells in the spiral ganglion of representative mice: a WT mouse at the age of 6.5 months (**a**), a VDR KO mouse at the age of 6.5 months (**b**), a WT mouse at the age of 10 months (**c**), and a VDR KO mouse at the age of 10 months (**d**). Caspase 3 activation was also detected in the stria vascularis of WT mice at the age of 6.5 months (**e**) and VDR KO mice at the age of 10 months (**f**). Scale bar = 9.6 μm.

Discussion

Most of the biological activities of 1α,25-dihydroxyvitamin D_3 require VDR, which translocates from the cytoplasm to the nucleus after ligand binding and then heterodimerizes with the retinoid X receptor [Mangelsdorf and Evans, 1995]. Our finding of VDR expression in the mouse cochlea implies that vitamin D is potentially important in maintaining hearing, which is in agreement with the previous reports [Brookes and Morrison, 1981; Brookes, 1983, 1985; Ziporyn, 1983]. Although VDR protein was not detected in the VDR KO mouse cochlea, which is at odds with reports on a truncated form of VDR in the same strain of VDR KO mice [Bula et al., 2005], this

Table 1. Caspase 3 activation in the spiral ganglion of WT mice and VDR KO mice

Group	Observed cells	Positive cells	Caspase 3 activation, %	Intensity of caspase 3 activation, AU
WT 6.5 months	157	65	41.40	1,538.45 ± 37.48
VDR KO 6.5 months	111	23	20.72	1,313.55 ± 12.44**
WT 10 months	189	80	42.33	1,212.83 ± 29.85
VDR KO 10 months	186	61	32.80	1,215.08 ± 12.80

** $p < 0.001$, statistically significant difference in caspase 3 activation intensity between VDR+/+ and VDR–/– mice at the age of 6.5 months. AU = Arbitrary unit.

can be explained by the difference in the epitope which was recognized by different antibodies. The antibody used in our study (Abcam, ab12129) was produced by inoculation with synthetic peptide corresponding to amino acids 5–19 of human VDR, whereas the antibody applied by Bula et al. [2005] was raised against a synthetic peptide mapping at the C-terminus of VDR of rat origin (Santa Cruz Biotechnology, sc-1008).

Our ABR results suggest that disruption of vitamin D-related bioactivity by partial deletion of the VDR gene accelerates age-related hearing loss. This auditory loss has been well documented in substrains of 129 mice [Ouagazzal et al., 2006; Zheng et al., 1999], and the finding that both WT and VDR KO mice show age-related hearing loss is also in agreement with the literature. However, there was a significant difference in threshold between young WT mice and young VDR KO mice, while there was no statistically significant difference between adult WT and VDR KO mice or between aged WT and VDR KO mice, indicating that a more rapid progressive hearing loss in mice was exaggerated by VDR mutation. The fact that there was no statistically significant difference between young VDR KO mice and adult VDR KO mice supports our hypothesis that age-related hearing loss developed earlier in VDR KO mice than in WT mice (before 6 months).

VDR exerts the biological role through regulating calcium and phosphate metabolism, and gene expression. The calcium and phosphate metabolism disorder can be compensated by feeding the animal a rescue diet, because the expression of Calbindin D 9K and plasma membrane calcium ATPase-1b (PMCA-1b) is VDR independent, whereas TRPV6 expression is VDR dependent [Bouillon et al., 2006; Song et al., 2003]. We were unable to restore the plasma calcium levels completely with the special diet, but the difference was rather small, which could also account for the hearing loss. Notably, TRPV6 expression in the brain is much higher than in the intestine, which may indicate that TRPV6 is a main player in brain calcium metabolism. Indeed, while TRPV6 decreased expression contributes to thalamic calcification [Nijenhuis et al., 2003] – a phenotype already reported in VDR KO mice [Kalueff et al., 2006a] – TRPV6 was not detected in the cochlea of either WT or VDR KO mice.

Analyzing our ABR data in VDR KO mice, we note that the elevated threshold and irregular ABR waveforms (except for wave I) can be explained as a result of decreased or abnormal brain stem neurons, since the cochlea, including the spiral ganglion cells, is normally developed. Deafness represents the late stages of neuronal degeneration, including large scale spiral ganglion cell loss (fig. 1i). Although 129S mice bear the potential of presbycusis, VDR KO can accelerate the process of aging as a consequence of downregulation of glial-cell-line-derived neurotrophic factor and nerve growth factor [Brown et al., 2003; Ohlemiller and Gagnon, 2004a, b; Ouagazzal et al., 2006; Taniura et al., 2006; Zhang et al., 2006; Zheng et al., 1999]. Thalamic calcification and degeneration may also induce brain stem lesions through the mechanism of retrograde degeneration [Kaga et al., 1999; Yamada et al., 2000].

Caspase 3 activation is also involved in age-related hearing loss and the mechanism of neuron degeneration may include both apoptosis and necrosis [Carloni et al., 2007; Riva et al., 2007]. Our finding of extensive activation of caspase 3 in the cochlea of WT 129S1 mice is in line with these reports. Both cytosolic and nuclear localization of cleaved caspase 3 protein in the cochlear cells indicates the role of caspase 3 activation in nuclear impairment [Kamada et al., 2005]. Except for the destroying effect, caspase 3 is also essential for the normal development of the auditory system [Morishita et al., 2001; Taka-

hashi et al., 2001], and its deficiency induces spiral ganglion degeneration and hearing loss in mice [Morishita et al., 2001]. While the mechanism sedimented from this phenomenon might be linked to caspase 3 regulating transcription through cleaving Ring1B in the nucleus [Wong et al., 2007], we also cannot rule out the possibility that decreased caspase 3 activation in VDR KO mice may be caused by the loss of some unknown gene expressions, which are controlled by VDR.

Several additional potential pathogenetic mechanisms have been tested in our study. TRPV4, a member of the TRP family, is a nonselective cationic channel that functions as a component of an osmotic/mechanical sensor [Liedtke et al., 2000, 2003]. Similar to TRPV4, the Drosophila protein Nanchung is essential for hearing in Drosophila [Kim et al., 2003]. TRPV4 mRNA and protein were also detected in murine cochlear hair cells, stria vascularis, and spiral ganglion cells, and hearing loss developed in TRPV4 KO mice [Liedtke et al., 2000; Shen et al., 2006; Tabuchi et al., 2005]. We studied the distribution of TRPV4 in the mouse cochlea using confocal microscopy. The expression pattern surrounding the endolymph edge also supports the hypothesis that TRPV4 is important in maintaining the fluid homeostasis of the endolymph, but this is not affected by VDR KO.

Potassium plays a significant role in maintaining the cochlear hair cell function. Connexin 26 is a fundamental structural protein in intercellular communication, especially K^+ circulation after the hair cell depolarization. Mutations in the GJB2 gene, which encodes the gap junction protein connexin 26, represent a major cause of prelingual, nonsyndromic, recessive deafness. These mutations are responsible for as much as 50% of such cases in many populations [Petersen and Willems, 2006]. We studied the connexin 26 protein in the mouse cochlea with immunofluorescent confocal microscopy and found that this fundamental structural protein was not affected by VDR KO.

Finally, inwardly rectifying K^+ channels regulate the resting membrane potential in many cells by contributing much of the resting K^+ conductance and maintaining low extracellular K^+ via spatial buffering mechanisms [Lagrutta et al., 1996; Reimann and Ashcroft, 1999]. Kir 4.1 (KCNJ 10) is expressed in the intermediate cells of the stria vascularis of the inner ear and helps to generate the endocochlear potential, which is the driving force of mechanoelectrical transduction by spatially buffering K^+ at a low level in a distinct intrastrial compartment [Hibino et al., 1997; Takeuchi et al., 2000]. Our results suggest that the system of generating the endocochlear potential was not disturbed by the VDR KO.

Conclusion

Our study shows that sensorineural hearing loss is progressively developed at an earlier age in VDR KO mice. Although the fundamental gene expressions in the cochlea were not influenced by VDR KO, caspase 3 activation disturbed by VDR genetic disruption may contribute to aberrant hearing phenotype in mice reported here.

Acknowledgements

We thank Prof. Shigeaki Kato (University of Tokyo, Japan) for providing the initial knockout mice for our research, Dr. Silvia Barth for her participation in the ABR measurements, and Mr. Peter Martinsson for his technical support on the confocal microscope. Special thanks to Mr. Justin L. LaPorte for his help with editing the manuscript, and to Ms. Marianne Kuuslahti for genotyping, paraffin embedding, sectioning, and peptide saturation assays. J.Z. was supported by an Identification of Environmental and Genetic Risk Factors for Age Related Hearing Impairment grant from the EU and the integrated EU project Nanoear (NMP4-CT-2006-026556). Y.Z. was supported by Nanoear. A.M., T.K., Y.R.L., A.V.K. and P.T. were supported by the EVO grant of Tampere University Hospital and the Academy of Finland. J.H.W. was supported by EVO (Tampere University Hospital) and DDGS (Turku).

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Journal of Steroid Biochemistry & Molecular Biology 104 (2007) 274–280
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Neophobia, sensory and cognitive functions, and hedonic responses in vitamin D receptor mutant mice☆

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Abstract

Vitamin D is a seco-steroid hormone with multiple actions in the brain, mediated through the nuclear vitamin D receptor (VDR). We have recently shown that mutant mice lacking functional VDR demonstrate altered emotional behavior and specific motor deficits. Here we further examine phenotype of these mice, testing their novelty responses, as well as cognitive and sensory (olfactory and gustatory) functions in the novel food, two-trial Y-maze and tastant consumption tests. In addition, we study depression-like behavior in these mice, using anhedonia-based sucrose preference test. Overall, VDR mutant mice showed neophobic response in several different tests, but displayed unimpaired olfactory and gustatory functions, spatial memory and baseline hedonic responses. Collectively, these data confirm that mutation of VDR in mice leads to altering emotional/anxiety states, but does not play a major role in depression, as well as in the regulation of some sensory and cognitive processes. These results support the role of the vitamin D/VDR neuroendocrine system in the regulation of behavior, and may have clinical relevance, enabling a better focus on psychiatric and behavioral disorders associated with dysfunctions in this neuroendocrine system.

Keywords: Vitamin D receptor; Anhedonia and depression; Y-maze; Olfaction; Gustation; Spatial memory; Neophobia

1. Introduction

The most active hormonal form of vitamin D is 1,25-dihydroxyvitamin D (calcitriol), which is implicated in both brain development and adult brain functioning [1–6]. Biological effects of this seco-steroid hormone are mediated through the nuclear vitamin D receptor (VDR), a ligand-activated transcription factor [7,8] widely expressed in the central nervous system [1,9–13]. Numerous brain disorders are linked to vitamin D deficits and/or VDR dysfunctions [13–15]. In both animals and humans, this hormone regulates behavioral and neuronal activity [13,16].

We have recently shown that mice lacking functional VDR (VDR mutant mice) display several behavioral abnormalities, including high anxiety and specific motor deficits [15,17], supporting the role of VDR in the regulation of behavior. In line with this, relatively high concentrations of VDR were found in the limbic system [1,9,10], the brain area that regulates emotional behaviors. Several other studies using VDR mutant mice [18,19] further strengthen the notion that vitamin D and VDR are involved in behavioral regulation.

Notably, high levels of VDR have been detected in multiple hippocampal areas [1,9–11]. As hippocampus is directly implicated in the regulation of cognitive processes, such as memory and learning, this raises the possibility that vitamin D and VDR may be involved in the regulation of these functions [2]. Indeed, while Altemus et al. [20] reported no significant impairment of cognitive functions in young adult rats deprived of vitamin D, transient prenatal vitamin D depletion has been found to affect learning and memory in rats [2]. To elucidate the role of VDR in cognitive functions further, the present study aimed to assess memory in VDR mutant mice in a two-trial Y-maze.

Since the assessment of general physiological status and sensory systems is a key part of behavioral phenotyping of

☆ Poster paper presented at the 17th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, 'Recent Advances in Steroid Biochemistry and Molecular Biology' (Seefeld, Austria, 31 May–03 June 2006).

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doi:10.1016/j.jsbmb.2007.03.032

mutant animals [21,22], these factors had to be considered in our VDR mutant mice. Indeed, several lines of evidence suggest that vitamin D and VDR may be involved in the regulation of sensory processes. For example, VDR is also widely distributed throughout the olfactory system [23], indirectly supporting the possibility that vitamin D and VDR may modulate olfaction. Likewise, VDR genetic partial ablation leads to hearing defects in mice [24], consistent with clinical data [25,26] on hearing deficits associated with vitamin D dysfunctions [27]. Other recent data have shown that in some tissues vitamin D up-regulates transient receptor potential (TRP) vanilloid calcium-selective cation channels, such as TRPV5 and TRPV6 [28,29]. Representing cellular sensors responding to temperature, touch, pain, osmolarity, taste and other stimuli [28,29], these vitamin D-regulated channels may underlie the role of this hormone in potential modulation of sensory pathways. Therefore, the second aim of the present study was to examine whether VDR gene mutation may affect the mouse sensory functions, such as olfaction and gustation.

Finally, the psychotropic mood-elevating effects of vitamin D have also been reported in the literature [14,30–35]. Significantly lower levels of vitamin D were found in psychiatric patients suffering from depression [33,36], and there was a predictable positive correlation between serum vitamin D levels and the reduction of affective symptoms [36,37]. From this point of view, it was interesting to assess depression-like phenotype in VDR mutant mice in the present study. Since specific motor deficits [17] preclude testing depression-like behavior in VDR mutant mice in several traditional depression tests (such as, the forced swim and tail suspension tests [21,22]), in the present study we subjected these mice to an anhedonia-based depression paradigm, the sucrose preference test [21,38]. This well-validated and popular depression test was chosen for its insensitivity to motor activity levels, and performed in the present study in conjunction with testing the mouse gustatory functions.

2. Material and methods

2.1. Animals

Subjects were adult (3–5 months) wild type (WT, +/+; $n = 10$) and VDR mutant (−/−; $n = 10$) male mice on 129S1 genetic background. The animals were littermates obtained by heterozygous crosses (for at least eight generations) from the VDR mutant mouse strain initially generated in the University of Tokyo, Japan [39]. Mice were maintained in a virus/parasite free facility on a 12-h light–dark cycle, and housed one to two mice per cage, with food and water ad libitum (unless noted otherwise). To normalize blood calcium levels, VDR mutant mice received a special diet (Lactamin AB, Sweden) containing 2% Ca, 1.25% P, and 20% lactose supplemented with 2.2 IU vitamin D/g. The genotype of the animals was confirmed by polymerase chain reaction (PCR) on DNA prepared from tail tissue. Primers were used to amplify a 400-bp Neo band and 166-bp VDR band from the targeted allele. All animal care and experimental procedures in this study were conducted in accordance with European legislation.

2.2. Apparatus and procedures

All behavioral tests were performed between 12.00 and 19.00 h in a dimly lit room. To assess the mouse working memory (Experiment 1), we used a two-trial version [40] of the Y-maze test, a traditional model of spatial working memory and exploration [13]. The Y-maze, made of Plexiglas, was elevated to a height of 70 cm and consisted of three walled arms (30 cm long, 10 cm wide, walls: 15 cm), radiating at an angle of 120° from each other. The study of spatial memory consisted of two Y-maze trials, with a 30-min interval between trials. During trial 1 (acquisition phase), one arm of the Y-maze (chosen randomly among the three arms) was closed, and remained unavailable for mice to explore. Each mouse was placed in one of the other two arms, and allowed to explore them for 5 min. The duration (s) and the number of visits (four-paw criterion) to the two arms were recorded by an experienced observer sitting 2 m away from the apparatus. The apparatus was thoroughly cleaned with ethanol solution after each animal. During trial 2 (retrieval phase), the animals had free access to all three arms, and the number and the duration (s) of visits to each arm were recorded for 5 min, as described earlier.

In a separate study (performed 2 weeks later, Experiment 2), we food-deprived the mice for 24 h, and then analyzed their spatial memory in the two-trial Y-maze with food reinforcement and a 2-h inter-trial interval. To minimize novelty factor in this experiment, the animals were allowed to explore the Y-maze freely for 10 min prior to testing. During the first trial, a 0.2-g pellet of familiar food (chow) was placed in a randomly selected arm, and the latency (s) to eat food, as well as the number of correct entries (arm with food), incorrect entries (empty arm) and total number of entries were recorded for 5 min. In addition, we calculated the percentage of incorrect entries (correct/total entries × 100%) for each animal in both trials. During trial 2, the location of food pellets was the same for each animal as during the initial trial. Food pellets were changed between animals and trials.

To study behavioral response to novel food (Experiment 3), we used a combination of familiar food versus novel food. A piece of white bread (2.3 g), vanilla (1.6 g) or onion (1.7 g) was used as novel food in this study. The protocol was as used previously in Experiment 2. Two weeks later, the animals were re-tested, using a combination of novel food (vanilla) versus familiar food. In the last test, performed 2 weeks later, a novel aversive food (onion) was given in combination with the familiar food (chow). The procedures were as described above, with a 4-h interval between trials. All food pellets were changed between animals and trials.

To determine whether the VDR mutant mice have altered olfactory function, we assessed their performance in the

buried food pellet test [41] (Experiment 4). Briefly, the mice were placed on a food-restricted diet (0.2 g standard chow per mouse) for 24 h. On the following day, a food pellet was buried approximately 0.5 cm deep under the bedding in a clean cage. The mouse was then placed in the diagonally opposite corner, and the latency (s) to find the buried food (as an index of olfactory function) was measured with a 5-min cut off time. The food was considered found if the animal was holding it in its forepaws (the mice were then allowed to consume food, and returned to their home cages). The bedding material was changed between animals.

To assess in parallel the mouse gustatory function and their baseline hedonic responses (Experiment 5), we used the two-bottle "preference" test [38], in which individually housed animals were given a free choice between two bottles (one with taste solution and another one with tap water). The solutions used in the present study included: sweet (1% sucrose, BDH Laboratory Supplies, England; 7 days), sour (50 mM citric acid 1-hydrate, Riedel-de Haen A.G., Germany), bitter (100 μM cycloheximide, Sigma–Aldrich, Germany), and salty (0.3 M NaCl, J.T. Baker, Holland), 4 days each. The position of bottles was switched every 24 h to avoid side preferences. The consumption of water and taste solutions was estimated simultaneously in both genotypes by weighing the bottles daily, and calculating daily preference (%) as total tastant solution/total water intake × 100%. No food or water deprivation was used in this experiment.

2.3. Statistical analyses

All results are expressed as mean ± S.E.M. Differences between genotypes were analyzed by the Mann–Whitney *U*-test. Difference between trials was analyzed using the Wilcoxon signed ranks test. A probability less than 0.05 was considered statistically significant in all tests.

3. Results

Table 1 summarizes the results of Experiment 1. In the spatial memory test, there were no genotype differences in the latency to enter the novel arm, the number of entries to the novel arm, and time spent exploring the novel arm.

In Experiment 2, using the Y-maze task with food reinforcement, there were also no genotype differences in the latency to eat in either trial (Table 2). However, during the first trial, the VDR mutant mice demonstrated significantly more "incorrect" entries to empty arms, and displayed more total entries, than did their WT littermates. In contrast, during the second trial, the number of entries was similar in both genotypes (Table 2).

Assessing behavioral responses to novel food (Experiment 3), we found no differences between the groups in the latency to eat bread on trial 1, whereas this measure on trial 2 was significantly shorter in the WT mice than in the mutant group (Table 3). Total number of entries was unaltered in both genotypes in all trials. As can be seen in Table 3, testing the mouse response to vanilla food revealed no genotype differences in the latency to eat on trial 1. In contrast, during the second trial, VDR mutant mice showed a shorter latency to eat compared to their WT controls. WT mice also displayed more entries to the food arm (correct entries) and to the empty arms (incorrect entries) during the initial trial, compared to the mutant group. In both trials, the WT mice demonstrated more total entries than did the VDR mutant mice (Table 3). Finally, in test with onion, the WT mice showed a longer latency to eat food, and made more entries, compared to the VDR mutant mice. Again, these behavioral parameters did not differ significantly between the genotypes during the second trial (Table 3).

In Experiment 4, we assessed olfactory functions in our mice, using the buried food pellets test. Overall, both geno-

Table 1
Behavioral performance (spatial memory and exploration) in the two-trial Y-maze (Experiment 1) in male wild type (WT) and vitamin D receptor (VDR) mutant mice

Habituation, 5 min	WT ($n = 10$)	VDR mutants ($n = 10$)	*U*-test
Latency (s) to enter arm 1	78.9 ± 25.8	92.3 ± 25.7	NS
Latency (s) to enter arm 2	62.0 ± 23.3	97.0 ± 29.5	NS
Number of entries to arm 1	10.4 ± 1.61	7.50 ± 1.46	NS
Number of entries to arm 2	11.0 ± 2.04	6.10 ± 1.52	NS
Number of total entries	21.4 ± 3.35	13.6 ± 2.90	NS
Test phase, 5 min (30 min interval between trials)	**WT**	**VDR mutants**	***U*-test**
Latency (s) to enter arm 1	89.0 ± 27.9	81.4 ± 26.1	NS
Latency (s) to enter arm 2	119 ± 37.2	148 ± 32.4	NS
Latency (s) to enter arm 3	99.4 ± 26.5	147 ± 29.3	NS
Number of entries to arm 1	6.10 ± 1.03	4.20 ± 0.63	NS
Number of entries to arm 2	3.70 ± 0.87	3.10 ± 0.62	NS
Number of entries to novel three arms	6.70 ± 1.37	4.70 ± 1.13	NS
Total number of entries	16.5 ± 3.02	12.0 ± 2.12	NS
Entries to novel arm 3 (%)	40.6 ± 45.5	39.2 ± 53.1	NS
Time (s) spent in novel arm 3	57.9 ± 13.3	52.9 ± 12.6	NS

Data are presented as mean ± S.E.M. Statistical significance was set at $P < 0.05$. NS: statistically not significant.

Table 2
Behavioral performance of male wild type (WT) and vitamin D receptor (VDR) mutant mice in the familiar food test (two-trial Y-maze with a 2 h inter-trial interval; Experiment 2)

	WT ($n=10$)[a]	VDR mutants ($n=10$)[a]	WT ($n=10$)[b]	VDR mutants ($n=10$)[b]	Between genotypes[c]		Between trials[d]	
					Trial 1	Trial 2	WT	VDR mutant
Y-maze + food pellets interval time 2 h								
Latency (s) to eat the food	19.3 ± 3.07	57.5 ± 21	46.1 ± 20.4	22.1 ± 4.43	NS	NS	NS	$P<0.04$
Entries to the food arm	1.00 ± 0.00	1.60 ± 0.16	1.00 ± 0.00	1.00 ± 0.00	NS	NS	NS	$P<0.014$
Entries to the empty arms	0.60 ± 0.22	3.00 ± 0.79	0.60 ± 0.22	0.30 ± 0.15	$P<0.02$	NS	NS	NS
Total number of entries	1.60 ± 0.20	4.60 ± 0.80	1.60 ± 0.22	1.30 ± 0.15	$P<0.02$	NS	NS	$P<0.011$
Incorrect entries (%)	26.7 ± 9.00	51.2 ± 9.60	26.7 ± 9.03	15.0 ± 7.64	$P<0.05$	NS	NS	$P<0.011$

Data are presented as mean ± S.E.M (NS: $P>0.05$).
[a] Trial 1.
[b] Trial 2.
[c] *U*-test.
[d] Wilcoxon signed ranks test.

Table 3
Behavioral performance of male wild type (WT) and vitamin D receptor (VDR) mutant mice in the novel food test (two-trial Y-maze; Experiment 3)

Habituation, 10 min	WT ($n=10$)[a]	VDR mutants ($n=10$)[a]	WT ($n=10$)[b]	VDR mutants ($n=10$)[b]	Between genotypes[c]		Between trials[d]	
					Trial 1	Trial 2	WT	VDR mutants
Food pellets + bread bread, interval time 2 h								
Latency (s) to eat the food	152.0 ± 35.0	156.0 ± 40.0	67.0 ± 17.0	194.0 ± 38.0	NS	$P<0.05$	NS	NS
Total number of entries	8.00 ± 1.00	9.00 ± 1.00	5.50 ± 1.20	9.00 ± 2.40	NS	NS	NS	NS
Incorrect entries (%)	47.1 ± 6.5	55.6 ± 1.7	52 ± 7.6	51.5 ± 9.1	NS	NS	NS	NS
Food pellets + vanilla interval time 4 h								
Latency (s) to eat the food	110.7 ± 27.3	96.7 ± 34.2	102.3 ± 29.2	53.7 ± 25.5	NS	$P<0.03$	NS	NS
Entries to the food arm	4.30 ± 0.70	2.40 ± 0.37	2.10 ± 0.59	1.10 ± 0.10	$P<0.03$	NS	$P<0.011$	$P<0.01$
Entries to the empty arms	5.60 ± 1.07	2.60 ± 0.37	4.20 ± 1.81	1.20 ± 0.36	$P<0.02$	NS	NS	$P<0.017$
Total number of entries	9.90 ± 1.72	5.00 ± 0.70	6.30 ± 2.35	2.30 ± 0.45	$P<0.02$	$P<0.02$	NS	$P<0.007$
Incorrect entries (%)	55 ± 3.1	52.00 ± 3.5 4	60.31 ± 4.28	43.33 ± 7.54	NS	NS	NS	NS
Food pellets + onion interval time 4 h								
Latency (s) to eat the food	233.1 ± 29.0	138.0 ± 32.7	277.0 ± 21.7	116.6 ± 36.4	$P<0.04$	$P<0.008$	NS	NS
Entries to the food arm	6.30 ± 0.90	3.30 ± 0.68	6.80 ± 1.08	2.60 ± 0.65	$P<0.03$	$P<0.01$	NS	NS
Entries to the empty arms	12.3 ± 1.58	5.60 ± 1.54	13.2 ± 2.13	3.80 ± 1.25	$P<0.007$	$P<0.002$	NS	NS
Total number of entries	18.6 ± 2.38	8.90 ± 2.16	20.0 ± 3.15	6.40 ± 1.78	$P<0.01$	$P<0.004$	NS	NS
Incorrect entries (%)	66.02 ± 1.96	56.94 ± 4.19	65.83 ± 2.23	55.6 ± 2.9	NS	$P<0.01$	NS	NS

Results are presented as mean ± S.E.M (NS: $P>0.05$).
[a] Trial 1.
[b] Trial 2.
[c] *U*-test.
[d] Wilcoxon signed ranks test between trials.

types showed similar latencies to find (WT: 55 ± 9 s; VDR mutant mice: 76 ± 16 s, NS) and eat (WT: 138 ± 32 s; VDR mutant mice: 83 ± 16 s, NS) familiar food, confirming unimpaired olfaction in VDR mutant mice.

This study of mouse gustatory function and baseline hedonic responses (Table 4; Experiment 5) showed no impairments in tastant discrimination in VDR mutant mice, as both genotypes demonstrated similar consumption of sweet, salt,

Table 4
Intake of different tastants in the wild type (WT) and vitamin D receptor (VDR) mutant mice (Experiment 5)

Taste solutions	WT	VDR mutants	*U*-test	Wilcoxon signed ranks test	
				WT	VDR mutants
1% sucrose	52.15 ± 1.99 ($n=9$)	60.22 ± 5.25 ($n=10$)	NS	NS	NS
0.3 M NaCl	28.99 ± 2.18 ($n=9$)	29.81 ± 6.74 ($n=7$)	NS	$P<0.008$	$P<0.018$
50 mM citric acid	46.27 ± 1.04 ($n=9$)	43.01 ± 2.59 ($n=10$)	NS	$P<0.018$	$P<0.001$
10 μM cyclohexamide	22.71 ± 1.05 ($n=9$)	20.22 ± 1.86 ($n=10$)	NS	$P<0.008$	$P<0.005$

Data are presented as mean ± S.E.M. (NS; $P>0.05$), calculated as the preference percentage (daily intake (%) = taste solutions daily intake/total liquid intake (taste solution intake + water intake) × 100).

sour or bitter solutions. All mice consumed markedly less NaCl solution (≈30%), whereas the intake of citric acid was close to 50% in both genotypes, with a similar (≈20%) aversion of cyclohexamide. Both genotypes showed unaltered intake of sucrose solution and water (Table 4).

4. Discussion

In general, the main findings of the present study can be summarized as follows: VDR mutant mice display unaltered spatial memory, olfaction, gustation and hedonic responses, but demonstrate aberrant responses to novel food in different contexts.

Despite the fact that numerous VDR were found in memory-controlling brain areas [10,11], and cognitive deficits were reported to be due to low vitamin D in both animals [2,20] and humans [35], our results showed that in Y-maze spatial memory task, both WT and VDR mutant mice explored novel arms equally well on both trials (Table 1). These findings are generally consistent with observations in VDR mutant mice tested in several other memory tasks, including spontaneous alternation and open field within-trial habituation [42,43]. Moreover, both genotypes demonstrated similar inter-trial habituation (reflecting their unaltered long-term memory) in the open field test [43]. Collectively, these and our present data support the notion that memory domain (unlike locomotion and anxiety [15,42]) is most likely unaffected by the VDR mutation in mice.

Importantly, the number of Y-maze arm entries in VDR mutant mice was similar or even higher (than in the WT mice) in some tests here (Table 2), indicating unimpeded general motor functions in these mice. Earlier studies have shown (rev. in: [44]) that increased motivation, such as hunger, may alter the animal exploratory performance in different tests, influencing both their cognitions and emotionality. In the Y-maze task with food reinforcement (Experiment 2), both groups of hungry mice showed similar ability to find familiar food in both trials, again suggesting that motor functions of VDR mutant mice in this test were relatively normal. However, the trial 1 data of this experiment, reporting higher locomotion in the VDR mutant mice, seem to be consistent with the above-mentioned effects of hunger on exploration, and may be explained by their somewhat higher initial stress–reactivity (compared to the WT mice), also in line with their increased anxiety phenotype (also see a tendency to a longer latency to eat food in trial 1, Table 2).

Since memory testing using familiar food as reinforcement (Experiment 2), revealed elevated trial 1 activity in the VDR mutant mice, we wanted to assess their responses to novel versus familiar food. As unfamiliar food is known to induce strong neophobic responses, the latency to consume novel food can be used as a measure of anxiety [45,46]. In Experiment 3, using white bread as a novel food, we noted that latency to eat was reduced in the WT mice on trial 2, but not in VDR mutant mice (showing a tendency to increase; Table 3). These observations suggest that upon second novel food exposure the VDR mutant mice were less willing to consume novel food—the response which may again be best explained by their high anxiety phenotype, as already reported [15].

Likewise, in tests with vanilla and onion, the VDR mutant mice showed reduced exploration—a profile generally seen as a sign of stress and anxiety in rodents [22]. Interestingly, the latency data from these tests yielded conflicting results, with no genotype difference on trial 1 for vanilla (resembling data from bread exposure), but not for onion, and shorter trial 2 latencies in the mutant group (Table 3). Although it is difficult to interpret such latency data, in our present study we aimed at a more accurate interpretation of mouse phenotypes, following recommendations to avoid conclusions based on a single measure [21], and trying to assess several different indices. It is also possible that varying latency responses in this experiment represent a complex interplay between different factors (not fully explored here), such as baseline anxiety, aversive/attractive properties of different types of food, neophopic responses, and the effects of repeated testing (including a combination of two trials, and a test battery involving several different food exposures). Importantly, as rich Ca "rescue" diet (used here in the VDR mutant mice) has been shown to normalize their plasma Ca levels [8,47], it was possible conclude that the behavioral differences reported here were not due to non-specific factors, such as hypocalcaemia. Olfactory functions of VDR mutant mice, tested here in the buried food test (Experiment 4), also appear to be unaltered, confirming that behavioral responses of these mutant mice in the novel food tests are not due to olfactory deficits.

Can genotype differences in gustatory functions affect the mouse behaviors in our study? Assessing the mouse gustatory function, we found no overt impairments in gustation in the VDR mutant mice, with a predictably strong aversion of salty and bitter solutions in all genotypes (Table 4). The fact that gustatory functions, including TRP-mediated perception of bitter taste [28,29] were normal in VDR mutant mice, suggests that VDR do not affect the TRPV system or other signaling pathways relevant to sensory mechanisms.

Finally, as human vitamin D deficiency has long been known to be accompanied by irritability, depression and psychoses [13,16], it was possible to expect altered baseline depression-like behaviors in mice lacking functional VDR. To the best of our knowledge, the present study was the first study assessing depression domain in VDR mutant mice. However, the VDR mutant mice showed unaltered hedonic responses (Table 4), negating the possibility that genetic defect in VDR *per se* may lead to anhedonic depression. While these data seem to contradict reports on antidepressant effects of vitamin D [14,31,32,34,35] it is also possible that such effects of vitamin D are not mediated through VDR, and may be due to its other (steroid-like) effects, resembling known antidepressant effects of other neurosteroids [48].

In conclusion, the results of the present study show that the VDR system is neither crucially important for cognitive

functions (such as memory), nor involved in the regulation of some major sensory functions. VDR genetic ablation is also unable to alter depression-like behaviors in these mice, as assessed by their unimpaired hedonic responses. However, our data demonstrate that VDR mutant mice consistently display neophobic anxiety-like responses to novelty in several different tests. Accompanied by normal motor activity levels in all these tests, our data confirm increased vulnerability to stress in VDR mutant mice, as has already been suggested [15].

Collectively, these data contribute to our understanding of the complexity of VDR mutant mouse behavioral phenotype, warranting their further in-depth analyses and the use in behavioral neuroscience as a potentially interesting genetic animal model of vitamin D-related brain disorders. These data may also be clinically relevant (enabling a better focus on human brain/behavioral disorders associated by vitamin D/VDR deficits), and contribute to the growing recognition of the importance of a neurosteroid hormone vitamin D in the regulation of brain functions and behavior [27,49].

Acknowledgements

This study was supported by research grants from the Medical Research Fund of Tampere University Hospital and the Academy of Finland. We thank Ms. Marianne Kuuslahti for her excellent technical assistance, and Mrs. Heini Huhtala for her advise on statistical analyses.

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