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

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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.

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Vitamin D is a neuroactive steroid hormone with multiple functions in the nervous system including the regulation of differentiation, Ca2+ 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, 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, on animals 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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Here, we tested the Tokyo VDR KO mice in the model of chemically induced seizures. Pentylenetetrazole (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 Tamperé, 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 06:00h. 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, post-partum pups were weaned and assigned to different cages based on their genotype and gender. The animals were experimentally naive 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. 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 ability to induce proconvulsions 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 Ca²⁺ 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 Tamperé. 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 Ca²⁺ levels and seizure measures was analysed using the Spearman rank-order correlation coefficient (R)

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 of the clonic seizures (P = 0.08, U-test) and higher mortality rate (P = 0.06, U-test).

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

<table>
<thead>
<tr>
<th>Measures</th>
<th>WT (n = 10)</th>
<th>VDR KO (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency to the first twitch (s)</td>
<td>66.9 ± 4.5</td>
<td>50.4 ± 4.4</td>
</tr>
<tr>
<td>Latency to orofacial seizure (s)</td>
<td>73.8 ± 5.3</td>
<td>63.2 ± 4.9</td>
</tr>
<tr>
<td>Latency to clonic seizure (s)</td>
<td>521 ± 220</td>
<td>179 ± 74a</td>
</tr>
<tr>
<td>Latency to tonic seizure (s)</td>
<td>701 ± 246</td>
<td>201 ± 80a</td>
</tr>
<tr>
<td>Latency to death (s)</td>
<td>1259 ± 226</td>
<td>429 ± 167</td>
</tr>
<tr>
<td>Number of mice with twitches</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Number of mice with orofacial seizures</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Number of mice with clonic seizures</td>
<td>8/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Number of mice with tonic seizures</td>
<td>7/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Mortality rate</td>
<td>6/10</td>
<td>9/10a</td>
</tr>
<tr>
<td>Duration of orofacial seizures (s)</td>
<td>9 ± 1.4</td>
<td>8.7 ± 1.6</td>
</tr>
<tr>
<td>Duration of clonic seizures (s)</td>
<td>10 ± 3</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>Duration of tonic seizures (s)</td>
<td>12 ± 4.6</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Total duration of seizures (s)</td>
<td>31 ± 5.8</td>
<td>35.7 ± 5.5</td>
</tr>
</tbody>
</table>

- a Robust trend (P = 0.05–0.08, U-test).
- * 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).
compared to the WT controls (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 behaviour 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 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 down-regulates 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 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 ground strains may influence mouse sensitivity to PTZ [13,36]; however, our present results using VDR KO mice are still interesting in this context. 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 (CS7Bl/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 biosynthesis [9]. The current evidence, in agreement with our previous findings [23,24], and recent studies [25,26], display 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 possible to assess the seizures in mice or rats chronically deprived of Vitamin D—a further tool to explore the potential role of Vitamin D in epilepsy. 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 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 and VDR in various types of epilepsy disorder. Moreover, it may also be feasible to assess the seizures in epilepsy. 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.

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