Thalamic calcification in vitamin D receptor knockout mice

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Vitamin D is a steroid hormone with many important functions in the brain, mediated through the nuclear vitamin D receptor. Here, we report that aging nuclear vitamin D receptor knockout mice demonstrate a symmetric thalamic calcification with numerous Ca/P-containing laminated bodies. These results are consistent with clinical findings showing brain calcification in patients with vitamin D deficiency. Our results suggest that nuclear vitamin D receptor deficiency leads to brain mineralization in vitamin D receptor knockout mice, which may represent an experimental model of intracranial calcification. *NeuroReport* 17:717–721

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Introduction

In addition to the regulation of mineral metabolism and cell differentiation [1,2], vitamin D plays an important role in the brain, including induction of many genes, modulation of neurotrophin release, neurotransmitter metabolism and neuroprotection [3,4]. Recent data show that vitamin D is synthesized and inactivated by the brain, further confirming its potential role as a novel neuroactive steroid or neurosteroid hormone [3–6].

The functions of vitamin D are mediated through the nuclear vitamin D receptor (VDR), a member of the steroid receptor superfamily [1,7]. Several recent clinical and experimental data outline the possible role of the vitamin D/VDR neuroendocrine system in the regulation of normal and pathological brain mechanisms, including brain differentiation and growth [3], neuroprotection [5], epilepsy [8] and behaviors [9,10]. Previous studies have confirmed that the VDRs are widespread throughout the brain in both animals and humans [11–13]. High concentrations of VDR were recently found in the basal ganglia, brain stem, hypothalamus and the limbic system, including the hippocampus and thalamus [12,13], outlining the potential biologically significant role of vitamin D in the brain.

Genetically modified animals provide a powerful tool for neurobiological research. Several mouse strains with genetically ablated VDR are currently available for biomedical research focusing on the biological functions of vitamin D and VDR [7,14]. These mutant mice develop rickets and osteomalacia, hypocalcemia, hypophosphatemia and hyperparathyroidism, thus representing an animal model of human hereditary vitamin D-resistant rickets [7,14]. In humans, such disorders have long been known to be accompanied by intracranial mineralization [15–19]. Here, we investigate the brain morphology of VDR knockout mice, examining whether genetic ablation of VDR may lead to its mineralization.

Methods

Adult male mice (20–25 g; University of Tampere, Tampere, Finland) were bred for this experiment from the strain initially generated in the University of Tokyo (Tokyo, Japan) [7]. The 129S1 mouse substrain was used as a genetic background. Mutant (−/−) mice were compared with the control wild-type (+/+) animals. The following age groups were analyzed in the present study: young adult (3–3.5 months, n=6 in each group), adult (4.5–6 months, n=5 in each group) and aged mice (9–14 months, n=4 in each group).

Tail clips were taken for genotyping performed using a polymerase chain reaction on DNA prepared from tail tissue. Four primers were used to amplify a 130-bp VDR band and a 150-bp Neo band from the targeted gene. On day 21 *post partum*, pups were weaned and assigned to different cages on the basis of their genotype and age. The mice used in the present study were maintained in groups of two to

three animals per cage in a virus/parasite-free facility (temperature 25±2°C, humidity 55±5%) and exposed to a 12:12 h light:dark cycle (lights on at 07:00 h), with food and water *ad libitum*. All animals were experimentally näve and were fed a special rescue diet containing 2% Ca, 1.25% P and 20% lactose (Lactamin AB, Kimstad, Sweden) known to reduce hypocalcemia and rickets in the VDR knockout mice [2].

Animals were killed with carbon monoxide, their brains removed, fixed for 24 h at 4°C in buffered 4% paraformaldehyde fixative, and then kept in 70% ethanol at 4°C until embedding in paraffin for neuromorphological examination. Coronal 5-μm sections were cut using a sliding microtome (Microm HM 430, Walldorf, Germany). Adjacent sets of sections from the same animals were stained using the hematoxylin–eosin (HE) and von Kossa silver nitrate methods for Ca salts. Both HE-stained and unstained microtome sections of brain tissue were examined to detect Ca and P using a scanning electron microscope (SEM, Philips XL 30, Eindhoven, Netherlands) equipped with an energy dispersive spectrometer (EDS, Model DX-4, EDAX International, Mahwah, New Jersey, USA), which utilizes x-rays emitted from the sample during bombardment by the electron beam, to characterize the elemental composition of the sample.

Capillary endothelial cells were demonstrated by using polyclonal rabbit antibody against human factor VIII antigen (DakoCytomation AS, Glostrup, Denmark). For immunohistochemistry, 3-μm paraffin sections were cut onto objective slides. Antigen retrieval for immunoperoxidase staining was performed on rehydrated sections in a microwave oven at 850 W for two 7-min cycles, using 10 mM Tris-ethylenediaminetetraacetic acid buffer (pH=9.0) as the retrieval solution. Immunostaining was carried out in a TechMate 500 Immunostainer using the EnVision polymer technique (DakoCytomation AS). Diaminobenzidine was used as a chromogen and hematoxylin as a nuclear stain. The specificity of immunohistochemistry was controlled by omitting the primary antibodies. This assay was performed in the same aged knockout mice (n=4) where brain calcification was observed. Known positive tissue samples were also used to confirm the staining reliability.

In addition, plasma Ca and P levels were measured in these mice using atomic absorption spectroscopy (Yhtyneet Laboratoriot, Helsinki, Finland). All animal experiments were performed in full compliance with the NRC Guide for the care and use of experimental animals, and approved by the Ethical Committee of the University of Tampere.

Results

Plasma Ca and P levels were affected in the aged VDR knockout group, showing a reduced Ca concentration (1.96±0.09 vs. 2.28±0.10 mmol/l, $P<0.05$, U-test) and a trend to a slightly elevated phosphate concentration (3.27±0.10 vs. 3.09±0.14 mmol/l, NS) compared with those in the respective wild-type controls (n=4 in each group). A similar trend was also observed in the younger age groups, including adult [mutants: Ca, 1.84±0.07; P, 5.29±0.56 mmol/l; n=4; wild type: Ca, 2.56±0.04 ($P<0.05$, U-test); P, 3.72±0.46 mmol/l; n=4] and young adult [mutants: Ca, 2.11±0.29 (NS); P, 4.32±0.58 mmol/l (NS); n=3; wild type: Ca, 2.54±0.08; P, 3.72±0.46 mmol/l; n=4] mice.

Figure 1 shows numerous concrements in brain tissue of the aged VDR knockout mice revealed by HE and von Kossa staining (n=4). These structures were located symmetrically in thalamic areas of the brain tissue, and not observed in the respective wild-type control mice (n=4). No such structures were observed in younger animals of both genotypes (n=4–5 in each group), suggesting that these bodies appear only in VDR-ablated animals in an age-dependent manner (Fig. 1a).

As can be seen in Fig. 1, these structures varied widely in size and shape, from small spheres or ovoids (20–30 μm in diameter) to large concrements greater than 400 μm. Most of these bodies had a laminated appearance (Fig. 1d). Rarely, they occurred around or within blood vessels, as further confirmed by the distribution of endothelial marker factor VIII (Fig. 1d and f). In addition, although we did not see signs of pronounced gliosis around the laminated bodies in the mutant mice, some increase in glial cells could be seen, especially around big laminated bodies (Fig. 1d). Notably, other brain areas were devoid of such deposits in any of the animals used here.

Basophilic hematoxylin and von Kossa staining (Fig. 1d and e) led us to assume that these laminated bodies found in the thalamus of the VDR mutant mice may represent an intracranial calcification. To examine this hypothesis in detail, the chemical composition of laminated bodies in brain tissue was identified by the SEM and EDS methods. The SEM micrograph for unstained sections of the brain tissue (Fig. 2a) showed numerous round particles in the brain tissue, resembling the laminated bodies seen in both HE and von Kossa stained slides. Figure 2b shows the EDS point analysis of the laminated body of the brain tissue indicating high concentrations of Ca and P in these laminated bodies. The average composition of the laminated body is approximately 55% Ca and 45% P, giving the molar ratio Ca/P of about 1.22, and thus most likely being Ca phosphate, the most common salt in calcified concrements observed in humans. Carbon and oxygen edges and small amounts of sodium and magnesium in the EDS spectrum were due to scattering from the surrounding matrix. The spatial distribution of the chemical composition was also confirmed with the x-ray mapping, which shows that Ca and P concentrate in the laminated bodies *per se*, and not in the surrounding matrix (Fig. 2c).

Discussion

Vitamin D plays a major role in Ca/P homeostasis in different tissues, especially in the bone, serum, muscle and intestine [1–3]. In humans, hypovitaminosis D associated with hypocalcemia and hyperparathyroidism has long been known to be accompanied by intracranial calcification (affecting the basal ganglia, cerebral cortex and cerebellum), partially relieved by vitamin D therapy [16–18]. In addition, calcium deposits were seen in the basal ganglia in patients with normal Ca/P levels but reduced plasma vitamin D levels [15], further strengthening the link between the abnormal vitamin D/VDR system and intracranial calcification.

Our study is the first report showing severe thalamic calcification in aged VDR knockout mice, suggesting that an impaired vitamin D/VDR system may affect mineral homeostasis in the mouse brain. The concrements observed here had a laminated appearance, showed no inflammatory response to mineral deposits and tended to occur near the blood vessels (Fig. 1), strikingly resembling brain

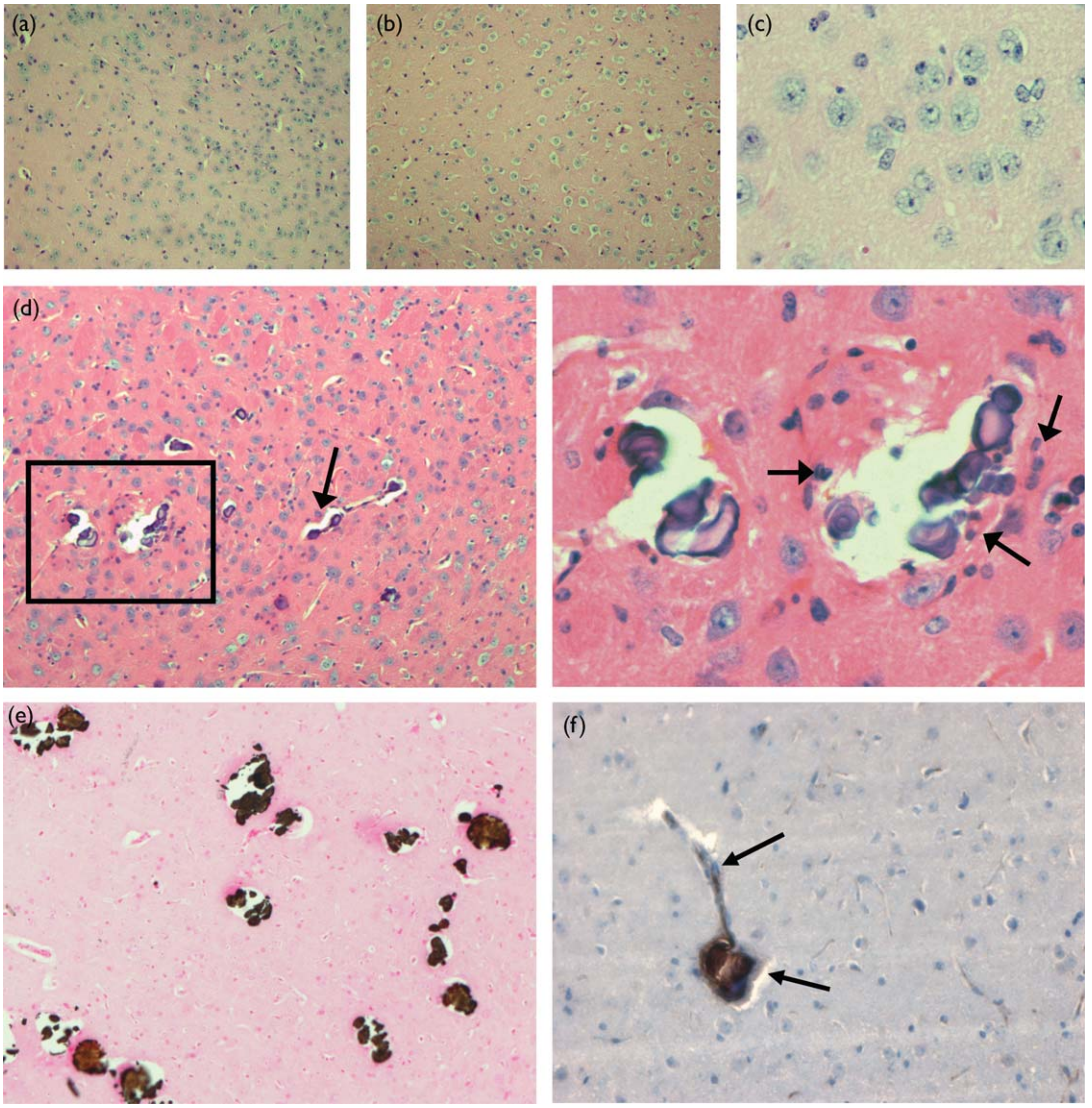

Fig. 1 Thalamic mineralization in representative aged nuclear vitamin D receptor (VDR) knockout (KO) mice. (a and b) Unaffected brains of adult and aging wild-type 129SI control mice; hematoxylin–eosin (HE) staining, original magnification × 10. (c) Unaffected brains of young adult VDR KO mouse; HE staining, × 40. (d) Numerous concrements in the thalamic area of aged VDR KO mice; HE staining. Note location of some concrements within a blood vessel (indicated by arrows, × 10). In an enlarged photo (left, × 40), see their laminated structure and the increase in glial cells (indicated by arrows) around them. (e) von Kossa staining showing basic (Ca) composition of these bodies (× 40). (f) Factor VIII staining confirming location of the concrements within the walls of blood vessels (indicated by arrows, × 40).

mineralization in humans [16–21]. Collectively, this suggests that aged VDR knockout mice may represent a useful animal model of vitamin D/VDR-dependent intracranial calcification.

Analyzing our data, several possible pathogenetic mechanisms have to be considered, especially the role of Ca. As all mouse groups used in this study received a similar (rich Ca) diet, the occurrence of thalamic calcification only in the aging VDR knockout mice (Fig. 1) suggests that this phenomenon is not due to excessive Ca supplementation. Lowered plasma Ca levels in these mice, however, imply that, as in humans [16], prolonged hypocalcemia may also play a pathogenetic role in brain calcification reported here. Moreover, vitamin D-dependent proteins calbindin-D9k and calbindin-D28k are the major proteins involved in the regulation of Ca homeostasis in various tissues [14,22]. Recent studies have shown that expression of calbindin-D9k (but not calbindin-D28k) is reduced markedly in the brain of VDR knockout mice, failing to normalize with a diet rich in Ca, lactose and P [15]. Overall, this suggests that genetic ablation of VDR, via both hypocalcemia and Ca-independent brain mechanisms, may affect intracranial Ca metabolism in mice leading to ectopic calcification reported here.

Assessing the role of age in the brain calcification here, we note that concrements were only seen in the aging (Fig. 1) but not in the younger VDR knockout mice. This observation is in line with clinical findings showing that brain calcification is most commonly found in patients older than 50 years [19,21]. Consistent with this, bilateral thalamic calcification was observed in aging rodents [23–27], suggesting that both VDR ablation and aging may interplay in provoking thalamic calcification observed in this study. To the best of our

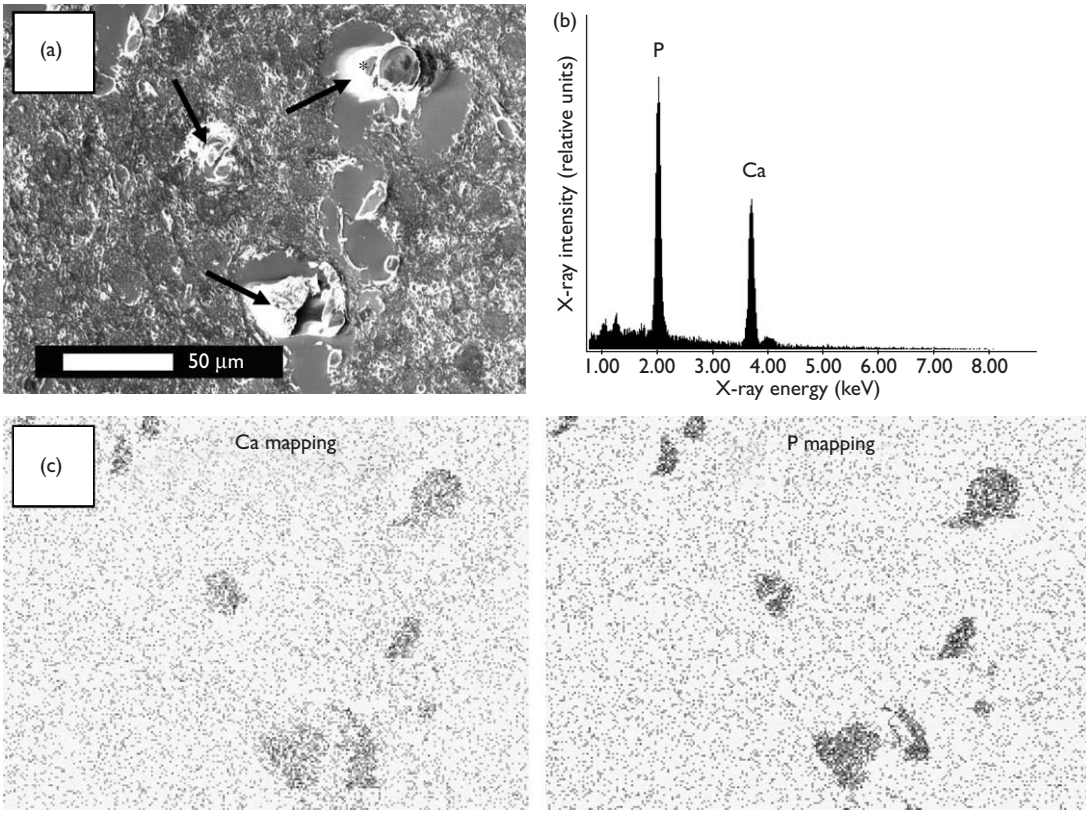

Fig. 2 Energy dispersive spectrometer (EDS) spectrum and x-ray mapping of an unstained nuclear vitamin D receptor knockout mouse brain slice (thalamic region), confirming the composition of its laminated bodies from Ca and P. (a) Scanning electron microscope (SEM) micrograph of the brain slice with several mineral concrements (indicated by the arrows). (b) EDS spot analysis of the representative laminated body [indicated by asterisk, (a)], identifying its elemental composition by collecting and plotting x-rays (emitted from the sample) by energy, and automatically identifying and labeling the elements responsible for peaks in the energy distribution (Ca, P). (c) The x-ray mapping showing the distribution of specified elements within the sample from (a). Elements (Ca, P) were chosen on the basis of EDS data. Note the overlapped Ca and P mapping of the concrements, confirming their being composed of these elements.

knowledge, however, there have been no published studies reporting spontaneous progressive calcification in the wild-type 129S1 mice, consistent with the lack of concrements in all wild-type control groups used in this study. This suggests that VDR genetic ablation *per se* (and not the age or genetic background of mice) is the factor responsible for the brain tissue calcification reported here.

Importantly, numerous recent data indicate that the vitamin D system is involved in neuroprotection mediated by VDR [3,5]. As genetic ablation of VDR clearly impairs such neuroprotective effects, this factor may also contribute to pathogenesis, further increasing brain calcification (already provoked by abnormal Ca homeostasis). Given the importance of neuroprotection for the aging brain, and the age-dependent nature of brain calcification, especially in the thalamus [23–26] (Fig. 1), this hypothesis seems indeed likely. Perhaps, this factor may explain why thalamic areas, and not other brain structures, were affected in the present study. In line with this, several clinical and experimental data [17,27] showed that thalamic calcification may be a marker of brain lesions and reduced neuroprotective mechanisms in the brain – an observation that may further underline robust calcification observed in the VDR knock-out mice here. Clearly, further studies are needed to understand both the pathogenetic mechanisms of such calcification, its neuroanatomical distribution and potential neurophysiological and motor–behavioral consequences.

Conclusion

Our results link progressive thalamic calcification in mice to genetic ablation of VDR, suggesting that the vitamin D/VDR dysfunctions may be involved in the formation of mineral (Ca/P) deposits in the brain tissue. Displaying concrements strikingly resembling intracerebral calcification in patients (including those with abnormal Ca and/or vitamin D levels [15]), these mice may represent a useful animal model of intracranial calcification, further confirming the importance of the vitamin D/VDR system in maintaining normal physiological functioning of the brain.

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